



UNIVERSITY  
OF TASMANIA

# Minimising the risk of norovirus contamination in Australian commercial oysters

By

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Submitted in fulfilment of the requirements for the Degree of

Doctor of Philosophy

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University of Tasmania. December 2015



# Table of Contents

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Table of Contents .....	iii
List of Figures .....	viii
List of Tables .....	x
Abbreviations .....	xi
Abstract .....	xiii
Declaration of Originality .....	xvi
Authority of Access .....	xvi
Statement of Ethical Conduct .....	xvi
Acknowledgements .....	xvii
Literature Review .....	1
1.1 Preface .....	1
1.2 Historical perspective .....	1
1.2.1 Oysters in Australia .....	1
1.2.2 Food poisoning .....	2
1.3 Norovirus .....	3
1.3.1 NoV structure .....	4
1.3.2 NoV diversity .....	5
1.4 NoV Disease .....	6
1.4.1 Infection .....	6
1.4.2 NoV illness .....	8
1.4.3 Prevalence of NoV illness in the community .....	8
1.4.4 Foodborne outbreaks of NoV illness .....	9
1.4.5 Foodborne NoV outbreaks linked to oysters .....	10
1.4.6 NoV isolated from shellfish .....	12
1.4.6 Secondary cases of NoV illness .....	13
1.5 Oysters .....	14
1.5.1 Oyster biology .....	14
1.5.2 NoV binding in oysters .....	15
1.5.3 Detection of NoV in oysters .....	16
1.5.3 Elimination of NoV from oysters .....	17
1.5.4 Inactivation of NoV in oysters .....	18
1.6 Shellfish Quality Assurance .....	18
1.6.1 Classification of harvest areas .....	19
1.6.2 Triggers for closure of harvest areas .....	21

1.7 Contamination of growing areas with NoV .....	22
1.7.1 STP effluent in oyster growing areas .....	23
1.7.2 Post harvest depuration .....	24
1.7.3 Alternative indicators of sewage contamination in oyster growing areas .....	24
1.8 Prevalence of NoV in oyster growing areas.....	25
1.9 NoV illness outbreaks and Australian oysters .....	26
1.10 Burden of NoV illness in Australia.....	26
1.11 Summary .....	27
1.12 Thesis Objective.....	28
1.12.1 Aim.....	28
1.12.2 Objectives.....	28
Chapter Two: Methods.....	29
2.1 Preface.....	29
2.2 Introduction.....	29
2.2.1 Method for NoV detection in oysters.....	29
2.2.2 HAV detection .....	30
2.2.3 Bocavirus: a potential alternative indicator for NoV .....	30
2.2.4 Methods Evaluated.....	31
2.3 Methods.....	32
2.3.1 NoV Detection and Enumeration.....	32
2.3.2 Detection of HAV in oysters.....	36
2.3.3 International Ring Trial/ proficiency testing.....	37
2.3.4 Sewage: sample preparation and RNA extraction .....	37
2.3.5 Virus sequencing.....	37
2.3.6 Methods for the detection of HBoV in SRO and sewage .....	38
2.3.7 Enumeration of <i>E. coli</i> in oysters.....	39
2.4 Results.....	39
2.4.1 NoV testing method in Australian oysters .....	39
2.4.2 HAV detection in oysters.....	44
2.4.3 Sewage and virus detection.....	44
2.4.4 Virus sequencing.....	45
2.4.5 International Ring Trial/ proficiency testing.....	45
2.4.6 Enumeration of <i>E. coli</i> in oysters.....	45
2.4.7 HBoV detection in oysters and sewage.....	45
2.5 Discussion .....	46
2.5.1 NoV testing method validation .....	46
2.5.2 Cloning and sequencing primer bias.....	47



2.5.3 HBoV in shellfish and sewage .....	47
Chapter 3: A sampling programme for an investigation of potential NoV contamination of oysters in a river estuary.....	49
3.1 Preface.....	49
3.2 Introduction.....	49
3.3 Methods.....	51
3.3.1 Sampling .....	51
3.3.2 Sampling events .....	52
3.3.3 Sample handling and transport.....	54
3.3.4 NoV testing .....	55
3.3.5 <i>E. coli</i> testing.....	55
3.3.6 Additional samples.....	55
3.4 Results.....	55
3.4.1 NoV detection .....	55
3.4.2 <i>E. coli</i> results.....	58
3.5 Discussion .....	61
Chapter 4 Prevalence of NoV in Australian oysters .....	63
4.1 Preface:.....	63
4.2 Introduction.....	63
4.3 Method .....	64
4.3.1 Shellfish sampling plans .....	64
4.3.2 Shellfish sampling sites and sampling programme.....	64
4.3.3 Shellfish transportation .....	68
4.3.4 Shellfish processing and molecular testing for NoV .....	68
4.3.5 Nucleotide sequence accession numbers .....	68
4.4 Results.....	68
4.4.1 NoV occurrence .....	68
4.4.2. <i>E. coli</i> results.....	68
4.4.3. Control data.....	69
4.4.4. Sequencing.....	69
4.5 Discussion .....	69
Chapter 5: Spatial and temporal distribution of NoV and <i>E. coli</i> in SRO following a sewage overflow into an estuary.....	73
5.1 Preface:.....	73
5.2 Introduction.....	73
5.3 Methods.....	74
5.3.1 Oyster sampling sites .....	77
5.3.2 Sample collection and transportation to the laboratory .....	78

5.3.3 Analysis of oysters and sewage for NoV and HAV .....	79
5.3.4 Sequencing .....	79
5.3.5 Enumeration of <i>E. coli</i> in oysters .....	79
5.3.6 Statistical analysis of results .....	79
5.4 Results .....	80
5.4.1 Sewage overflow and hydrology .....	80
5.4.2 NoV and HAV occurrence .....	83
5.4.3 <i>E. coli</i> results .....	85
5.4.5 Sequencing .....	86
5.5 Discussion .....	87
Chapter 6: Evaluation of risk management of Australian oyster harvest areas for the protection of oyster consumers from NoV illness .....	91
6.1 Introduction .....	91
6.2 Review of potential sources of NoV contamination in Australian oyster harvest areas .....	91
6.2.1 On-site sewage management systems .....	94
6.2.2 Sewage Treatment Plants .....	98
6.2.3 Marine vessels .....	102
6.3 Extent and effectiveness of current risk management practices .....	104
6.3.1 Conclusions of Hay, McCoubrey and Zammit .....	105
6.4 Theoretical contamination events .....	107
6.4.1 Hypothetical event of single episode of diarrhoea .....	107
6.4.2 Event of a sewage overflow into a harvest area .....	108
6.4.3 Probability of detecting NoV in a batch of contaminated oysters .....	109
6.5 Additional risk mitigation strategies to protect the oyster consumer .....	110
6.5.1 Risk mitigation strategies for the prevention of NoV contamination .....	110
6.5.2 Risk mitigation strategies for the early detection of NoV contamination .....	113
6.6 Recommendations and Summary .....	117
Chapter 7: Discussion and future directions .....	119
7.1 Summary and Conclusions .....	119
7.2 Future directions .....	122
Bibliography .....	125
Appendix A.1 New South Wales Oyster Sampling Plan .....	153
Appendix A.2 Oyster Sampling Plan for South Australia .....	161
Appendix A.3 Oyster Sampling Plan for Tasmania .....	167
Appendix A:4 Oyster testing for Chapter 3. Results of <i>E. coli</i> and NoV GI & GII Testing .....	173
Appendix A:5 .....	178
Appendix A:6 Chapter 5. Results for individual PCR results in genome copies/ g shellfish gut .....	179

Appendix A:7 Best practice regulatory approaches to onsite sewage system management (293) .. 180

Statements of Authorship and Published papers ..... 180

# List of Figures

---

<b>Figure 1.1:</b> Diagram of NoV genome organization .....	4
<b>Figure 1.2:</b> Oysters, showing anatomical components of the Pacific oyster. ....	14
<b>Figure 1.3:</b> Photograph showing a shucked oyster and a dissected oyster gut. ....	17
<b>Figure 1.3:</b> OSMS near to waterway and oyster harvest area.....	23
<b>Figure 2.1:</b> C <sub>T</sub> of NoV GI RNA standards for each qPCR run. Runs conducted after 29 <sup>th</sup> July 2010 were performed in a Rotorgene Q (Qiagen). A new set of NoV GI standards were prepared and used from run FB 27.....	40
<b>Figure 2.2:</b> C <sub>T</sub> of NoV GII RNA standards for each qPCR run. Runs conducted after 29 <sup>th</sup> July 2010 were performed in a Rotorgene Q (Qiagen). A new “low” standard was prepared from run FB 56b onwards. ....	41
<b>Figure 2.3:</b> qPCR results for known NoV GII positive faecal samplesl showing C <sub>T</sub> vs dilution of faecal samples. Three serial dilutions of each sample were tested. ....	42
<b>Figure 2.4:</b> qPCR amplification profiles for NoV GI.Positive results were recorded and represent from left to right, FC,GI H, GI M and GI L NoV GI RNA standards, undiluted Korean oyster sample (KO) and 1:10 KO .....	44
<b>Figure 2.5:</b> Photograph of a gel showing A and C primer, PCR products of 153 (HBoV2) dilutions 10 <sup>5</sup> to 10 <sup>9</sup> ; BR and UL oyster extract dilutions to 10 <sup>3</sup> . Ladder in 100bp gradations. ....	46
<b>Figure 3.1:</b> Diagram of Kalang River estuary and sampling sites .....	54
<b>Figure 3.2:</b> qPCR amplification profiles (normalised fluorescence vs. cycle number) for NoV GI.Faecal RNA control (FC), RNA standards High, Medium, Low and R3, site 5 samples 5 and 3. ....	56
<b>Figure 5.2:</b> Photographs of shucked SRO (Left) at installation, (Right) day 5 from site 1. SRO were healthy after 80 days at site 1.....	82
<b>Figure 5.3:</b> Scatter plot of individual qPCR test results as log <sub>10</sub> NoV GII copies/g shellfish gut versus distance (m) from the sewage overflow on day 5 for sites 1 - 4. The dashed line represents the RLOD (260 NoV GII copies/ g shellfish gut). Test results noted in blue and situated on the dashed line were ≤RLOD (not detected). <b>X</b> were detects ≤ RLOD, marked on the dashed line.....	83
<b>Figure 5.4:</b> Scatter plots of individual qPCR test results as log <sub>10</sub> NoV GII copies/g shellfish gut versus time (days) after the sewage overflow for each sampling site 1-6. Four individual qPCR test results were recorded for each sample collected from sites 1-5 and two for site 6 due to the number of spaces in the Rotorgene Q. The finely dashed line represents the RLOD (260 NoV GII copies/ g	

shellfish gut). Test results noted in blue and situated on the dashed line were  $\leq$ RLOD (not detected).

**X** were detects  $\leq$  RLOD, marked on the dashed line..... 84

**Figure 5.5:** Scatter plots of MPN  $\log_{10}$  *E. coli* /100g shellfish versus time (days) after the sewage overflow for each sampling site. The upper dashed line indicates the upper limit of detection (18,000 MPN/100 g shellfish). The lower dashed line indicates the lower limit of detection (20 MPN/100g shellfish). ..... 86

**Figure A.5:** Calculations of NoV GI RLOD.  $C_T$  of NoV GI RNA standard Low diluted to 1/10.. 178



# List of Tables

---

<b>Table 1.1:</b> Number of harvest areas and classifications by production region, as at August 2014. ....	20
<b>Table 2.1:</b> Detection of NoV in oyster and faecal samples with different levels of contamination..	43
<b>Table 3.1:</b> River Estuary Environmental Data for 2009.....	53
<b>Table 3.2:</b> Timetable and NoV detections in SRO samples from 24 <sup>th</sup> July 2008 to 17 <sup>th</sup> September 2009 .....	57
<b>Table 3.3:</b> NoV GI detections and <i>E. coli</i> MPN for SRO sampled at site 1 .....	59
<b>Table 3.4:</b> Timetable and <i>E. coli</i> detected in SRO collected during sampling events in 2009. ....	60
<b>TABLE 4.1:</b> Location and parameters of oyster sampling sites for the study .....	66
<b>TABLE 4.2:</b> MPN of <i>E. coli</i> / 100 g of shellfish detected in oysters sampled seasonally from each growing area.....	67
<b>Table 5.1:</b> Estuary characteristics.....	77
<b>Table 5.2:</b> Calendar of events: rainfall; freshwater inputs; and sewage overflows in estuary .....	81
<b>Table 5.3:</b> Salinity and water temperature for Council sampling sites A, B & 6.....	82
<b>Table 6.1:</b> Potential sources of NoV contamination as observed in six selected sanitary surveys. ..	93
<b>Table 6.2:</b> Regulations governing OSMS in NSW, TAS and SA. ....	96
<b>Table 6.3:</b> Government regulations requiring notifications of sewage spills impacting harvest areas to State shellfish authorities. ....	100
<b>Table 6.4:</b> Sewage overflows occurring in the six harvest areas investigated for this profile and the notifications recorded by the respective shellfish authorities .....	101
<b>Table 6.5:</b> Published data of selected outbreaks of NoV illness linked to Australian oysters. ....	104

# Abbreviations

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ASQAP	Australian Shellfish Quality Assurance Programme
CI	Confidence Limit
HAV	Hepatitis A virus
HBGA	histo-blood-group-antigen
ID <sub>50</sub>	50% infectious dose
ILOD	lower limit of detection
RLOD	Reliable limit of detection
Native oyster	<i>Ostrea angasi</i>
NoV	norovirus
NSSP	National Shellfish Sanitation Programme (USA)
NSW	New South Wales
NSWFA	New South Wales Food Authority
ORF	open reading frame
OSMS	On-site management system
P oyster	Pacific oyster ( <i>Crassostrea gigas</i> )
PCR	polymerase chain reaction

RdRp	RNA dependent RNA polymerase
SA	South Australia
SASQAP	South Australian Shellfish Quality Assurance Programme
SRO	Sydney Rock Oysters ( <i>Saccostrea glomerata</i> )
STP	Sewage Treatment System
SWD	stormwater drain
TSQAP	Tasmanian Shellfish Quality Assurance Programme
uLOD	upper limit of detection
VP1	norovirus major capsid protein
VP2	norovirus small capsid protein
UV	Ultraviolet
FC	faecal controls, mixed NoV GI/GII faecal suspension



# Abstract

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Outbreaks of human gastroenteritis caused by norovirus (NoV)-contaminated oysters occur worldwide and have periodically been linked to the consumption of Australian oysters. Contamination with NoV can occur when human excrement (faeces and vomit) containing the virus flows into oyster growing areas. Minimal data about the occurrence of NoV in Australian oysters was available at the commencement of this thesis due to the expense involved in testing oysters for viruses because no Australian commercial laboratories had the capacity to perform the test. To that time, all Australian samples were tested in New Zealand.

The aim of this Thesis was to establish an informed strategy for minimising the risk of NoV in commercial Australian oysters. To collect the NoV data, a sensitive and accurate method to detect NoV was adopted in this study and validated for Australian oysters and conditions.

Epidemiological evidence suggests that the occurrence of NoV in Australian oysters is low; therefore a sampling regime for the detection of NoV at low occurrence and concentration in oysters was designed. Using this sampling programme, NoV was detected in oysters (8.3%, n = 163) from a harvest area that had been associated with 2 NoV illness outbreaks. The results showed the need for a comprehensive sampling regime to ensure the detection of NoV.

To address the lack of systematically collected data on the occurrence of NoV in Australian growing areas and due to the impending imposition of international standards for NoV in Australia, a survey was conducted. This involved the collection of oysters from two geographically distinct oyster growing areas on four occasions from each of the three main oyster producing States in Australia and testing samples for NoV and *E. coli* (n = 120). The growing areas selected were considered by Australian shellfish authorities to be the most compromised in those States with respect to the potential for human faecal contamination, as identified by shoreline surveys.

NoV GII was detected in two samples (1.7%) but NoV GI was not detected. Some of the samples were found to have more than the guidance concentration of 230 *E. coli* per 100 g of shellfish flesh but these samples did not contain detectable concentrations of NoV. These results reinforce epidemiological data suggesting that NoV contamination of commercially produced Australian oysters is rare.

There is minimal data about NoV dispersal in waterways following sewage overflows. The persistence of NoV in Sydney Rock oysters (SRO) following sewage overflows was also unknown. These knowledge gaps were addressed by strategically placing SRO in an estuary downstream from a sewage pump station known to overflow periodically and initiating sampling after an overflow event. After the event NoV GII was detected up to 5.29 km downstream and persisted in SRO closest to the pump station outfall for 42 days. NoV GII concentrations decreased significantly over time; a reduction rate of 8.5% per day was observed in oysters located at two sites near the outfall ( $p < 0.001$ ). Five days after the overflow, NoV GII concentrations were found to decrease significantly as a function of distance at a rate of 5.8% per km ( $p < 0.001$ ). The decline in *E. coli* concentration with distance was 20.1% per km ( $p < 0.001$ ). NoV GI and Hepatitis A virus were not detected. A comparison of NoV GII reduction rates from oysters over time, derived from this study and other, published, research collectively suggest that GII reduction rates from oysters may be broadly similar, regardless of environmental conditions, oyster species and genotype.

In the final phase of the project, an investigation of risk management options for Australian commercial oyster harvest areas to protect oyster consumers from NoV was conducted. The result, a culmination of data and research, is a combination of strategies recommended for minimising the risk of NoV prevalence in Australian oysters. It was found that the risk is real, as demonstrated by the occurrence of 1-2 outbreaks of NoV illness associated with oysters annually. This was supported by the findings of a NoV contamination rate of 1.7% of oysters from Australian growing areas. Recommendations were made for risk management strategies to prevent contamination of oyster growing areas and to enable rapid detection and notification of contamination events when they occur.

It was found that communication between local councils, water utility operators and shellfish authorities regarding reporting sewage spills and the condition of potential sources of sewage spills (*i.e.* on-site sewage management systems (OSMS)) was lacking. A theoretical NoV contamination event in oyster growing areas was considered, showing that it was possible for a small human faecal mishap to cause illness in oyster consumers. Recommendations for a risk-based virus monitoring programme are proposed, including: regulation of OSMS by local councils; workshops on water quality to stimulate increased communication between Environmental Health Officers and shellfish authorities; upgrading sewage treatment plants where the effluent flows into oyster growing areas; mandatory dye studies of STP effluent flows and potential sources of sewage overflows for each growing area to enable more effective

closure times and delineation of the impacted area closed for harvesting; ASQAP to include guidance to put local management committees in place for each growing area, ASQAP to include guidance for portable toilets to be on oyster harvesting boats, and the development of an alternative indicator for NoV other than faecal coliforms *i.e.* bacteriophage testing.

The results of this Thesis will improve NoV risk management strategies used by shellfish authorities to protect the Australian oyster consumer and help to ensure that future control measures are commensurate with the risk associated with the growing area rather than requiring mandatory end product testing regimes for all areas.

# Declaration of Originality

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"This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright."

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# Authority of Access

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# Statement of Ethical Conduct

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The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University.

Felicity A. Brake, 28<sup>th</sup> December 2015

# Acknowledgements

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Hooray!

I have been extremely fortunate in studying with and learning from people who are passionate about their work, and focus for worthwhile outcomes for Australia and beyond. I have gained much from their dedication. Thank you Cath McLeod, Tom Ross, Andreas Kiermeier, Brenda Hay, Anthony Zammit, Rod Ratcliff and Andy Pointon.

To my supervisors, Cath McLeod and Tom Ross, thank you for your constant support and encouragement.

I am pleased with what I have achieved for the Shellfish Industry and couldn't have done this without financial support from the Australian Seafood CRC (scholarship), MISA, UTAS, NSWFA, Bellingen Shire Council, NSW Farmers Assn., NSW Oyster Industry, TSQAP, SASQAP and SARDI. Oysters were collected from the Brunswick estuary using a NSW I&I Permit No. P09/001-2.0 as per Section 37, Fisheries Management Act 1994.

I've had help from some amazing people within the Industry, thank you: Geoff Lawler for your help in the field, setting up sampling sites and sampling oysters; Bob Drake OAM and ACA Aquaculture for sampling oysters; Brenda Hay-AquaBio, (NSWFA) Anthony Zammit, Phil Baker and Grant Webster, who also helped in the field and with the bioaccumulation studies; Tony Troup, (TSQAP) - Ray Brown and Ali Turnbull who advised on sample site selection; (SASQAP) Ken Lee and Clinton Wilkinson who advised on sample site selection and helped with bioaccumulation studies; Geoff Holds (SARDI) for enumerating the *E. coli* in oysters; Lina Landinez and Kate Hodgson (SARDI) for conducting the HAV assays; Peter Rees (Byron Bay Council) for advice on sewage treatment; David Allsop (Manly Labs) for tidal data from Kalang River; Rod Ratcliff and Jane Arthur (SA Pathology) for their advice and provision of HBoV reagents.

My special thanks go to: my friends from SARDI, Geoff, Gayle, Viv, Sutasinee, Damien, Judy; and Tom Madigan whose help and humour especially over the last part of my candidature has been invaluable. To those who have supported me forever (which is a long time): Margie and Gini, their partners Steve and Tony and families; Kathleen and Warwick; Jo; the tennis gang & co., Linda, Ken, Jan, Paul, Christine, Lyn, Alex, Cathy, Margarete, Chris, Linda and Mark and their families. To my family, my number one special person Richard, my beautiful children now

amazing adults....(long thesis)...Charlie and Fergus; to Mum, Melinda, Greg, Fiona and Guy and the 'cuzs, thank you for supporting me.

I would also like to acknowledge and thank the traditional owners and their ancestors of Country where the oysters were sourced. These are the Kunarra Kuna group of the Pyemmairrener people (Tasmania), the Moomairremener people (Tasmania), the Dharug and Dharawal peoples (New South Wales) and the Barngarla and Nauo peoples (South Australia). Whilst at SARDI I worked on Kaurnu Land and would like to thank the Kaurnu people as traditional owners of the Land for this privilege.

Felicity

# Literature Review

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## 1.1 Preface

The chosen topic of study for this Thesis is “Minimising the prevalence of norovirus in Australian commercial oysters”. To introduce the areas studied in the Thesis, this Chapter presents background information about norovirus (NoV) and oysters and how they interact to cause illness in the oyster consumer.

## 1.2 Historical perspective

### 1.2.1 Oysters in Australia

Australians have been consuming shellfish for the past 40,000 years. Indigenous Australians left their shells in piles or middens beside their camps near the sea, rivers and lakes. The earliest middens at Willandra Lakes in New South Wales (NSW) have been estimated at 40,000 years old (1). Before British colonization, oysters were found in the coastal waters virtually all the way around mainland Australia. It is believed that coastal Aboriginal communities farmed oysters by establishing shell culch beds for oysters to attach to, in shallow areas of estuaries. After British colonization, both native oysters (*Ostrea angasi*) and Sydney Rock oysters (*Saccostrea glomerata*) (SRO) were harvested for food and their shells used for lime production. Naturally growing oysters were prolific in NSW estuaries in the mid nineteenth century, where areas of up to 100,000 m<sup>2</sup> were covered in SRO. The Oyster Culture Commission of 1877 reported “up to 18 mature oysters and spat in an area of 5 square inches”. However, harvesting oysters for lime production drastically reduced these numbers (2).

From 1880, oyster stock of *Ostrea angasi* and *Saccostrea glomerata* were translocated for fattening from New Zealand to the Australian east coast estuaries prior to sale in Australia. It is believed that the introduction of this stock resulted in mudworm disease (2). Between 1880 and 1900, mudworm permanently destroyed the remaining natural sub-tidal east coast oyster reefs (2) and in the late 1800’s the remaining naturally-growing oyster stocks were over-exploited and fished out in the States of Victoria, South Australia (SA) and Tasmania (TAS).

Australian commercial oyster farming began in NSW in the 1870’s once farmers had started to use racks and subtidal cultivation for SRO. Pacific oyster (*Crassostrea gigas*) farming started in

TAS in the 1960's and in SA in the 1970's (3). The industry has since evolved to farmgate sales of 100 million dollars (12530 tonnes) annually (4)

### 1.2.2 Food poisoning

One of the earliest journal reports of food poisoning associated with oysters was in the British Medical Journal in 1890. Cameron (5) reported that a number of people in Dublin who had consumed oysters for lunch had fallen ill with diarrhoea and vomiting. By 1897, the American Public Health Association had adopted a standard procedure for the coliform test, which was used to ensure that drinking water was free of faecal matter. The understanding that sewage could impact on the health of oyster consumers is seen as early as 1909 in Belling (6) who wrote:

**“Water Pollution.** The sanitary condition of the marketed shellfish taken from contaminated waters is not only at present to some extent endangering the public health, but is placing an undeserved stigma upon a most reputable and valuable source of food supply for the public. The public should demand laws closing, after proper scientific investigation, these polluted areas, and conferring the power to thoroughly enforce such laws. The danger arising from contamination should be reduced to a minimum by prescribing some definite regulations for transferring shellfish from these polluted waters to places free from contamination, where the shellfish may in brief season be rendered fit for the market.”

Sixteen years later, there was a massive outbreak of typhoid linked to oysters from Long Island, New York, involving 1500 cases and 150 deaths. The sale of oysters plummeted. As a consequence, the oyster industry lobbied the US government and as a result, the National Shellfish Sanitation Programme (NSSP) was introduced to provide public health assurances for the interstate shipment of oysters and the protection of both sellers and consumers.

In Australia, there is little documentation of illness associated with Australian oysters until a notable NoV outbreak in 1978 ( $n > 2000$ ) linked to oysters from the Georges River, NSW (7, 8). Following this and other outbreaks also associated with NSW oysters, depuration was mandated for oysters from NSW prior to sale (9, 10). Viral illness outbreaks linked to oysters from NSW continued to occur over the next decade, including a large outbreak in 1996 of Hepatitis A virus (HAV) following consumption of oysters from Wallis Lake ( $n = \text{approx } 500$  cases and one death) (11). TAS was the first State to introduce a shellfish quality assurance programme (TSQAP) in the 1960's that adhered to NSSP principles. An Australia-wide programme, the Australian Shellfish Quality Assurance Program (ASQAP), was introduced in 1988. The



Georges River harvest areas in NSW voluntarily adopted a safety programme in 1990 in response to problems in meeting statutory microbiological limits and to restore customer confidence in their product (12) and by 1997 the NSW shellfish quality assurance programme was formally implemented. Over the period 2001 - 2010, the number of outbreaks due to contaminated oysters decreased tenfold due to the introduction of ASQAP (13).

HAV incidence in Australia decreased from 120 per 100,000 people in 1960 to 1.1 per 100,000 people in 2007 due to improved sanitation and the introduction of vaccination programmes for Australians at high risk of HAV infection (14).

There have been no further outbreaks of HAV illness linked to Australian oysters since 1996, however, NoV illness outbreaks continue to occur at a rate of approximately one to two per year (15). The NoV illness outbreak in 2013 was linked to oysters from Tasmania, and involved 525 cases across three States.

To understand why NoV illness outbreaks continue to occur from consumption of Australian oysters we need to understand the virus, the oyster, their interaction and the major factors that contribute to contaminated oysters being harvested and consumed.

### **1.3 Norovirus**

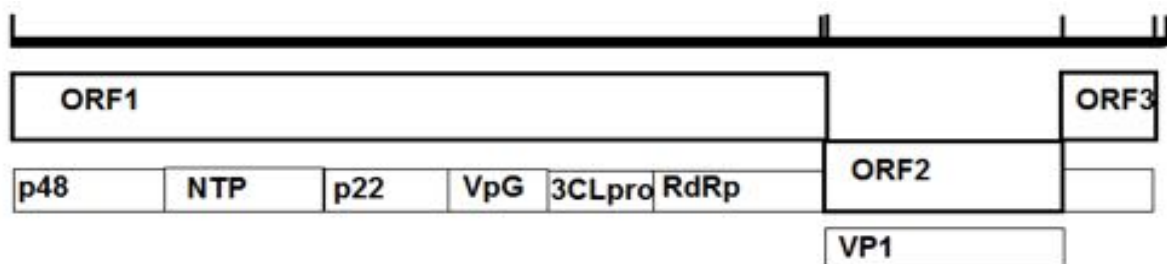
NoV are the major cause of both sporadic and epidemic gastroenteritis in humans and foodborne viral gastroenteritis outbreaks worldwide and the incidence is rising (16-18). It has been proposed that NoV are the perfect pathogen (19). The viruses enter the body through the faecal-oral route, are highly infectious, constantly evolving, environmentally robust and excreted in human vomit and faeces at extremely high concentrations (18).

The illness, also known as ‘winter vomiting disease’, was first described by Zahorsky in 1929 (20). It wasn’t until 1972 that the etiological agent, a 27 nm particle, was identified in a stool filtrate derived from an outbreak of acute infectious nonbacterial gastroenteritis in Norwalk, Ohio (21), whence it took its name. Norwalk virus has since been classified as NoV GI.1. NoV is a member of a highly diverse genus of the *Caliciviridae* family. Human NoV has been cultured in B cells *in vitro* at low levels, however with the aid of *Enterobacter cloacae* expressing histo-blood-group-antigens (HBGA), infection was significantly enhanced. It has been proposed that B cells (*in vivo*) are a cellular target of NoV, and enteric bacteria are a stimulatory factor for NoV infection (22). To date, no small animal models have been used for growing NoV. The lack of such models and practical cell culture models have made it difficult to elucidate details about

virus viability or to use molecular mutational analysis to study the function of viral proteins in infectivity or replication.

### 1.3.1 NoV structure

NoV are small and nonenveloped with single, positive stranded RNA of 7.4 to 7.7 kbases. The genome contains 3 open reading frames which encode for structural and non-structural proteins (23). ORF1 encodes the non-structural proteins, ORF2 encodes the major capsid protein (VP1) and overlaps the 3' end of ORF1 by 17-20 bases depending on the genogroup and ORF3 encodes a small capsid protein (VP2) (Figure 1.1).



**Figure 1.1:** Diagram of NoV genome organization

#### 1.3.1.1 Structural proteins

The NoV capsid is an icosahedron and is assembled from 90 dimers of VP1, the major capsid protein and one or two copies of VP2 the minor structural protein (24).

VP1 plays a regulatory role in the NoV replication cycle (25). One hundred and eighty VP1 molecules assemble into a T=3 icosahedral virion. There are two major domains, a highly conserved S-N terminal 225 amino acids and P protruding domain, containing a moderately conserved flexible P1 and a protruding P2 sub domain. P1 and P2 interact to increase the stability of the capsid and form protrusions on the virion. The hypervariable region of P2 is responsible for the antigenicity of the virus and plays a role in receptor binding and immune reactivity. It is likely to be responsible for the ABO HBGA interactions associated with susceptibility to NoV (26). It has been proposed that the conformational flexibility of the P domain enables NoV to dissociate from salivary mucin-linked HBGA and reassociate with HBGA linked to intestinal epithelial cells on the way to the gastrointestinal tract. Cross-competition assays and mutational analyses showed evidence for at least three distinct antigenic sites in the P domain and one in the S domain (27). The P2 sequence in GII has changed over time, with insertions and deletions, which has allowed the genotype to persist using receptor

switching and antigenic drift. Whereas, GI P2 encodes a surface-exposed conserved domain which limits changes in sequences and amino acids: persistence of the GI genotype is due to receptor switching and possibly host original ‘antigenic sin’ [when the body uses immunological memory based on a previous infection] (28).

VP1 can be expressed in insect cells and assembled into NoV-like particles (VLP) and has been used for research into antigenic relationships between viruses and carbohydrate ligand binding specificities. Dika *et al.* (29) compared electrochemical properties of MS2 phage and VLP and found that the presence of RNA in the virion contributes to the binding properties of the virus, which suggests that VLP may not be appropriate for predicting the behaviour of pathogenic viruses in aqueous media.

VP2 is a minor structural protein, not necessary for virus-like particle assembly but needed for production of infectious virus (30, 31). The protein shows extensive sequence variability between strains (32). VP2 binds to the VP1 at a site in the highly-basic interior surface of the capsid (the shell ‘S’, domain of VP1): the amino acids in this area of the S domain are tightly conserved across NoV which suggests a potential role in assisting capsid assembly and genome encapsidation (33).

#### **1.3.1.2 Non-structural proteins**

ORF1 encodes a large polyprotein that is processed by 3C-like protease 3CL<sup>pro</sup> into six non-structural proteins (Figure 1.1). These are p48, N-terminal protein, NTPase, p22, VpG, 3CL<sup>pro</sup> and RNA dependent RNA polymerase (RdRp). RdRp is thought to determine replication efficiency and genetic diversity, as it controls the rate new sequence is introduced into the genome (28).

#### **1.3.2 NoV diversity**

More than 40 strains of NoV have been isolated. These have been divided into 6 genogroups, GI-VI (34). Strains from genogroups I, II and IV infect humans, with GII being the most prevalent. Genogroups differ in their capsid sequence by more than 60% (35).

There is further diversity within genogroups that are subdivided into genotypes based on sequence analysis of regions in RdRp or VP1. Currently there are 9 GI genotypes and more than

30 GII genotypes. Genotypes are divided into strains or variants. Strains within a genotype exhibit 69-97% nucleotide similarity (36).

Recombination within genogroups can also occur around the ORF1/ ORF2 intersect, with recombinants sharing capsid or polymerase sequences, *i.e.* GII.4/ GII.12, which has a GII.4 RdRp and a GII.12 capsid. The recombinants are derived from pools of circulating NoV and provides a further mechanism for immune evasion (37). Recombination is also common within GI genotypes and depending on the genotype, is associated with periodic shifts in nucleotide sequences or circulating NoV GI (38).

NoV infect a broad range of species including pig (GII.11, GII.18, GII.19) (39, 40), lion, dog, cat (GIV) (41-43), cattle (GII) (44) sheep (GII) (40) and mice (GV) (45). Porcine and bovine NoV have been detected in shellfish taken from growing areas near intensive cattle farms (46). Bovine NoV GIII has been shown to bind to Gal HBGA epitope which is not present in oysters and humans do not express this particular glycan ligand (47), suggesting that it is unlikely to cause infection in humans. None of the NoV that infect animals have been shown to infect humans although there have been rare detections of human NoV in animals (48).

## **1.4 NoV Disease**

### **1.4.1 Infection**

NoV is highly infectious with the ‘50% probability of infection’ dose (ID<sub>50</sub>) calculated to be 18 virus particles (49). The virus can survive: freezing; temperatures up to 60°C; on surfaces for two weeks; in sediment for 6 months; and remain infectious in groundwater for at least 61 days (50, 51).

The virus is spread by droplets, person to person and through environmental contact. Infection depends on the presence or absence of carbohydrates which are HBGA, expressed on mucosal surfaces. HBGA act as binding ligands and putative receptors for NoV. Binding to specific HBGA varies between NoV strains. Expression of HBGA in humans relies on the presence of a functional FUT2 gene. Twenty percent of people do not have a functional FUT2 gene and are considered non-secretors: these people are resistant to GI.1 infection (52). There are however, other NoV genotypes that do infect non-secretors, possibly by attachment to Lewis carbohydrates (53). It is not yet known how infection progresses, however in 1961 Springer *et al.* (54) showed that humans have immunoglobulin (IgM) antibodies against non-self HBGA's

which were attributed to the presence of enteric bacteria with blood group activity, and in 2013, Miura *et al.* (55) showed that human faecal isolate *Enterobacter cloacae* that had HBGA activity, adsorbed NoV. This and the cultivation of NoV in human B cells in the presence of *Enterobacter cloacae* with HBGA activity (22), suggests that commensal microbiota in the intestine affect the likelihood of infection with human enteric viruses.

NoV GII.4 strains predominate in the community because they have a large cohort of people to infect, binding to HBGA A, B and O secretors who are found in 80% of the population. GII.4 was associated with the most person-to-person outbreaks (72%) in USA over a 4 year period and has been the cause of at least 4 global epidemics of gastroenteritis from 1995-2011 (16, 56, 57), although it is associated with fewer foodborne outbreaks than other genotypes (58). For a discussion of NoV foodborne outbreaks see S 1.4.4.

GII.4 have been circulating for more than 30 years (59) and have evolved by antigenic drift, driven by protective human herd immunity (60). They appear to evolve in a linear fashion without reversion to previous amino acid coding.

GII.3 strains have also been evident for 30 years (61). They are less prevalent in the community and have a lower tendency than GII.4 to infect adults (62). In oligosaccharide-based binding assays GII.3 strains bind HBGA types A and B strongly and Le<sup>b</sup> weakly whereas GII.4 binds A, B, H3, Le<sup>b</sup> and Le<sup>y</sup> (53). The fewer HBGA's bound may reflect fewer hosts whom are susceptible to GII.3 infection. In contrast to GII.4, GII.3 strains appear to revert to amino acid coding, with current strains showing similar amino acids to strains isolated 30 years ago (61).

NoV GI has been documented in the community since the detection of Norwalk virus (GI.I) in 1972 (21). NoV GI outbreaks are less common than NoV GII outbreaks (63-65). Bruggink *et al.* (38) showed that GI outbreaks were more likely to occur in non-healthcare settings (37.7%) than GII outbreaks (9.5%). The genotype has been shown to be more prevalent in waterborne outbreaks (66, 67) and linked to a higher proportion of shellfish related outbreaks than GII in the northern hemisphere (68). This may reflect the stability of the virus in water (50) and a differential accumulation efficiency of GI in binding to human-like HBGA in oysters (68). Genetic evolution of GI.2/ GI.2, GI.2/ GI.6 and GI.4/ GI.4 NoV occurs as a periodic shift in the nucleotide sequence, whereas GI.3b/GI.3 NoV circulate simultaneously (38).

The duration of immunity to NoV has previously been estimated at between six months and two years, using mathematical modelling of data from volunteer challenge studies of NoV GI.1 (Norwalk), GII.3 and GII.4. Simmons *et al.* (69) incorporated mathematical modelling of

community NoV transmissions with volunteer studies data and calculated the duration of immunity (for GII.4) as 4.1 – 8.7 years.

#### **1.4.2 NoV illness**

The illness is self-limiting and mild in most cases. The incubation period has been estimated at 1.2 days (70). The illness is characterized by nausea, vomiting, diarrhoea, and abdominal pain. Occasionally, headache and fever occur. In high-risk groups, symptoms can be severe and lead to dehydration and hospitalization. Death associated with NoV is observed to occur in 1.01 % of cases. The most affected are the elderly, with 90% of deaths in USA estimated to occur in the > 65 y age group (71, 72).

Studies of the clinical disease in volunteers have shown that, in an acute infection, the virus can be shed prior to symptoms being evident in 30% of cases and for up to 28 days after infection (73). In chronically-infected immunocompromised patients, the virus can be detected for months (288 days) and be infectious for at least 17 days post infection (74). Chronically infected and immunocompromised cases can act as a reservoir for the emergence of new viral variants, whereas the genetic makeup of the virus throughout the acute infection is highly homogenous and relatively stable (59).

Asymptomatic excretion is common: 12 % (age adjusted) of people had levels of NoV (20% in winter) which were detected using real-time PCR (75), though the study authors acknowledged that a small proportion of these may have had presymptomatic shedding.

It is interesting to note that murine NoV (MuNoV) has been observed to repair damaged mouse gut after chemical or antibiotic treatment and it has been proposed that the virus may replace the beneficial function of bacterial commensals in the mouse gut in supporting intestinal homeostasis and shaping mucosal immunity (76). In spite of the high prevalence of NoV in the community whether under asymptomatic or symptomatic conditions, the action of NoV in the human gut is yet to be explained.

#### **1.4.3 Prevalence of NoV illness in the community**

The estimated incidence of the disease in the general community varies considerably from country to country depending on how disease data are gathered. As the illness is mild and short-lived, most people with symptoms don't seek medical attention. A telephone survey about gastroenteritis conducted in Australia showed that approximately 1 in 5 people with gastroenteritis sought medical attention (77). In the UK and The Netherlands the incidence was

estimated from the detection of NoV in patient stools and the use of multipliers, at 4,500 and 3,800 cases per 100,000 person-years respectively (78, 79). The incidence estimate for USA *i.e.* 6500 cases per 100,000 person-years was calculated using data including faecal specimen submissions and national health care utilization rates (80).

Australian estimates of 1.5 million cases of NoV gastroenteritis among a population of ~22 million in 2010, *i.e.* 1 in 15, incorporated data from the telephone study, outbreak data and multiplication factors from the UK study. The 90% confidence limits were 1.22 – 1.94 million cases (81). Under-reporting correction factors for NoV gastroenteritis vary from 12.7 in Wales and England (82) to 1.76 in Germany (83). Complicating epidemiological studies further, sporadic cases of NoV gastroenteritis are generally not notifiable or reportable diseases unless contracted as a foodborne illness in an institution *e.g.* elderly care, school or hospital, or in an outbreak setting (usually 2 or more epidemiologically-linked cases). In Germany, laboratory-confirmed NoV is reportable, although this requires actions of firstly seeking medical attention (approximately 1 in 5 cases seek medical attention) followed by diagnostic testing (approximately 1 in 5 presented cases are tested). The annual incidence of reported NoV illness for 2011 in Germany was 142 cases per 100,000 (84), which is approximately 3% of the estimated incidence of NoV illness in the United Kingdom.

#### **1.4.4 Foodborne outbreaks of NoV illness**

In Australia foodborne illness is estimated to cost \$1.2 billion annually (85). In 2010, 25 percent (4.1 million cases) of an estimated 15.9 million cases of gastroenteritis were due to contaminated food. Kirk *et al.* (86) estimated that there were 15.9 million gastroenteritis cases in Australia in 2010, of these, 12.8 million (80%) were of unknown aetiology and 3.3 million of these were estimated to be foodborne. Similarly each year in the USA the causative organism of 38.4 million cases of foodborne acute gastroenteritis (80%) is unknown (87). Approximately, 276,000 of all gastroenteritis cases of known aetiology (35%) were caused by NoV-contaminated food. This was calculated from outbreak data and the multipliers used because of under-reporting of illness as mentioned above (86). In 2010 the New Zealand Food Safety Authority used a multiplier of 1:1000 to reported foodborne illness cases in order to calculate the estimated total number of foodborne NoV illness cases (88). It is notable that foodborne outbreaks have the highest illness burden of all NoV outbreaks (66).

Food can become contaminated with NoV at all stages of the food supply chain during production from irrigation water or growing water containing the virus or from food handlers

infected with the virus. The virus does not multiply in food but remains viable for extended periods of time on, or in, the food (89). The main food commodities implicated in NoV contamination from polluted irrigation or growing waters are foods that are eaten raw (90), such as bivalve molluscs, leafy greens, soft fruits (*i.e.* berries and tomatoes) (89, 91, 92). Ready-to-eat foods and foods that are eaten raw can be contaminated by food handlers with virus from vomit or faeces on their hands. Baert *et al.* (93) reported 80% of foodborne NoV outbreaks in Belgium were linked to food handlers. In 2010, seven of eight NoV outbreaks (117 cases) in Australia, were caused by infected food handlers. No outbreaks of NoV associated with Australian bivalve molluscs were recorded for 2010 (94).

Foodborne outbreaks of NoV illness are notifiable events in Australia (except in TAS) and most other countries. Nonetheless, under-reporting of the illness is a common problem, as discussed above.

For outbreak data collection in Australia, a foodborne gastroenteritis outbreak is considered to be when two or more associated cases of diarrhoea and/or vomiting occur in a 24h period with consumption of a common source of food or water, or that the pathogen has been confirmed from either a faecal or vomitus specimen or the food. Laboratory confirmation is used for NoV. Once the pathogen has been identified, in some cases from a single sample, no further laboratory testing is required, unless the illness symptoms change in patients diagnosed as positive (95).

#### **1.4.5 Foodborne NoV outbreaks linked to oysters**

The public health record in Australia suggests there have been few, and mostly sporadic, illness outbreaks linked to contaminated Australian oysters in the past decade (15, 96, 97). In the USA from 2009 to 2012, 19% of 1008 foodborne NoV outbreaks associated with a single food source were due to contaminated molluscs (98). A review of international foodborne NoV outbreaks in 2000-2008 ( $n = 40$ ) attributed a similar amount (17.5%) to bivalve shellfish (93). In the EU in 2013, 34 outbreaks of calicivirus (including NoV) were associated with shellfish, crustaceans, molluscs and associated products (99). In New Zealand between 2002-2009, shellfish were implicated in 34 of 1206 outbreaks of NoV gastroenteritis (100). In the period 2000-2011 OzFoodNet identified 18 NoV outbreaks that were epidemiologically linked to Australian oysters (15).

To give perspective to the number of illness outbreaks due to contaminated oysters, Australians consumed approximately 20 million dozen Australian produced oysters in 2013 with 97 percent of these purchased shucked and in the half shell (4). These were consumed raw or eaten partially



poached. Approximately 43-50 percent of households surveyed in Melbourne (n= 1248) ate oysters at least once a year, with 10-13 percent of those households, eating oysters at least once a month (101).

The detection of human strains of NoV in the oysters sampled from growing areas indicates that human excrement has polluted the area. NoV is not the only viral pathogen that may be present in faeces: aichivirus, sapovirus, astrovirus, rotavirus, adenovirus and HAV have also been detected in oysters following sewage overflows (102). Aichivirus, sapovirus, astrovirus and rotavirus cause similar gastroenteritis illness to NoV.

In a nine month study of volunteers consuming depurated oysters from the Georges River, Australia in 1978-9, 18.3% became ill (n=127) after one particular meal. Electron microscopy revealed a 27-30 nm Norwalk virus in five faecal extracts and a 22-25nm parvovirus-like particle in six extracts, with one extract containing both particles. A further three of nine people did not show symptoms after eating the oysters, but the smaller particle was detected in their faeces (103). NoV was detected in eight out of ten faecal samples taken from the Tasmanian cases (n = 306) in an outbreak linked with Tasmanian oysters (n=525) mentioned previously (97). All cases had symptoms of NoV illness and had consumed the oysters from the company involved.

NoV may not be the culprit in all cases of illness associated with sewage contamination, especially in view of the minimal laboratory diagnosis of the virus. However, it is the pathogen most likely to cause illness because of the potentially high load of NoV in sewage. Up to  $10^9$  virus particles/ L have been detected in sewage influent (104).

In 2013, a multistate NoV outbreak of 525 cases occurred which was linked to Tasmanian oysters. Cases were identified by:

- i) interview following alerts to Doctors and hospital emergency departments in Southern Tasmania,
- ii) media releases requesting possible cases to contact the Public Health Hotline, and
- iii) follow-up of contacts of self-reported cases notified to public health authorities.

Of the 306 cases occurring in Tasmania, ten faecal samples were tested and eight were confirmed positive for NoV. Of the 306 cases, 120 were probable cases. “Suspected” cases totalled 178, the definition for which was “eaten oysters from Company A, had NoV illness but not been interviewed by epidemiologists to ascertain specific details of their possible exposure”

(97). In the 2013 outbreak (n=525 cases in total), there were a total of 17 confirmed NoV positive faecal samples Australia-wide (97).

The path described above, which culminates in the determination of the number of cases associated with an outbreak of illness linked to NoV contaminated oysters is clearly fraught with difficulty for a number of reasons:

- i) it is difficult to detect and link hotspots of gastroenteritis to a particular food: the Tasmanian outbreak involved patients spread over a distance of 2500km, across four States and four separate Health departments;
- ii) the known reluctance of victims to report gastroenteritis (77);
- iii) notifying the public via the media to let them know that there were others with stomach upsets and encouraging them to report; followed by interviewing potential cases to determine whether the oysters were from a particular growing area (in some cases oysters from different sources were mixed at point of sale) and whether the gastroenteritis did in fact match NoV illness;
- iv) obtaining faecal samples – in some cases victims were identified and contacted many days after the initial onset when faecal concentrations of NoV may have been lower.

The low number of confirmed positive faecal samples (17) and two faintly positive oyster samples detected from 525 cases indicate how difficult it is to record outbreaks of illness related to oysters.

#### **1.4.6 NoV isolated from shellfish**

Vega *et al.* (56) reported that over a four year period in the USA, 16.1% of the outbreaks studied were foodborne and were mostly associated with GI.3, GI.6, GI.7, GII.3, GII.6 and GII.12 (56). Shellfish associated outbreaks have been shown to have a different frequency of NoV genotypes to that of other foodborne outbreaks (68, 100) which may be related to ligands in the shellfish which bind to NoV (68, 100). Le Guyader *et al.* (68) reported that GI genotypes were more frequently linked with shellfish associated NoV outbreaks, specifically GI.1, GI.4 and GI.2. Genotype GII.4 was the most frequently reported GII being linked with shellfish. Multiple genotypes of NoV are often detected in shellfish linked outbreaks, reflecting the multiple sources of the virus (102, 105).

In the southern hemisphere, NoV outbreaks linked to shellfish appear to occur in a different pattern. In New Zealand, GI was detected in only three outbreaks and GII in 29 outbreaks

between 2000-2009. Of the NoV detected in shellfish linked to outbreaks, two (from two separate outbreaks) were sequenced and found to be GII.4, and seven (linked to seven separate outbreaks) were found to be GII.c/GII.12. (100). The most recent outbreaks linked to Australian oysters featured GII.1 (TAS) (97) and GII.4 (NSW) (106) which were isolated from faecal specimens not from the oysters. Low virus concentrations hampered isolation and genotyping from oyster samples (97, 106). Outbreaks of NoV GI gastroenteritis are rarely observed in Victoria and SA (38) and (R. Ratcliff SA Pathology, SA June 2009 *pers. comm.*). The infrequency of GI outbreaks may explain the low number of GI outbreaks linked to shellfish in Australia.

Concentrations of NoV in oysters linked to outbreaks ranged from 100 – 16,000 genome copies / g shellfish gut (102, 105, 107). Thebault *et al.* (108) calculated the infectivity of NoV from oyster related outbreaks and produced ID<sub>50</sub> estimates for GI and GII of 1.6-7.1 genome copies per oyster consumed and a probability of infection  $p_m$  for a single NoV genome copy near 0.5. This is comparable to the ID<sub>50</sub> of 18 virus particles calculated by Teunis *et al.* (49) for NoV GI.6, the original Norwalk virus 8fIIa from faecal extract consumed by volunteers. The similarity in ID<sub>50</sub> may be due to the impact of HBGA and other binding sites in the oyster that recognize and bind NoV resulting in infectious NoV in the shellfish gut.

#### **1.4.6 Secondary cases of NoV illness**

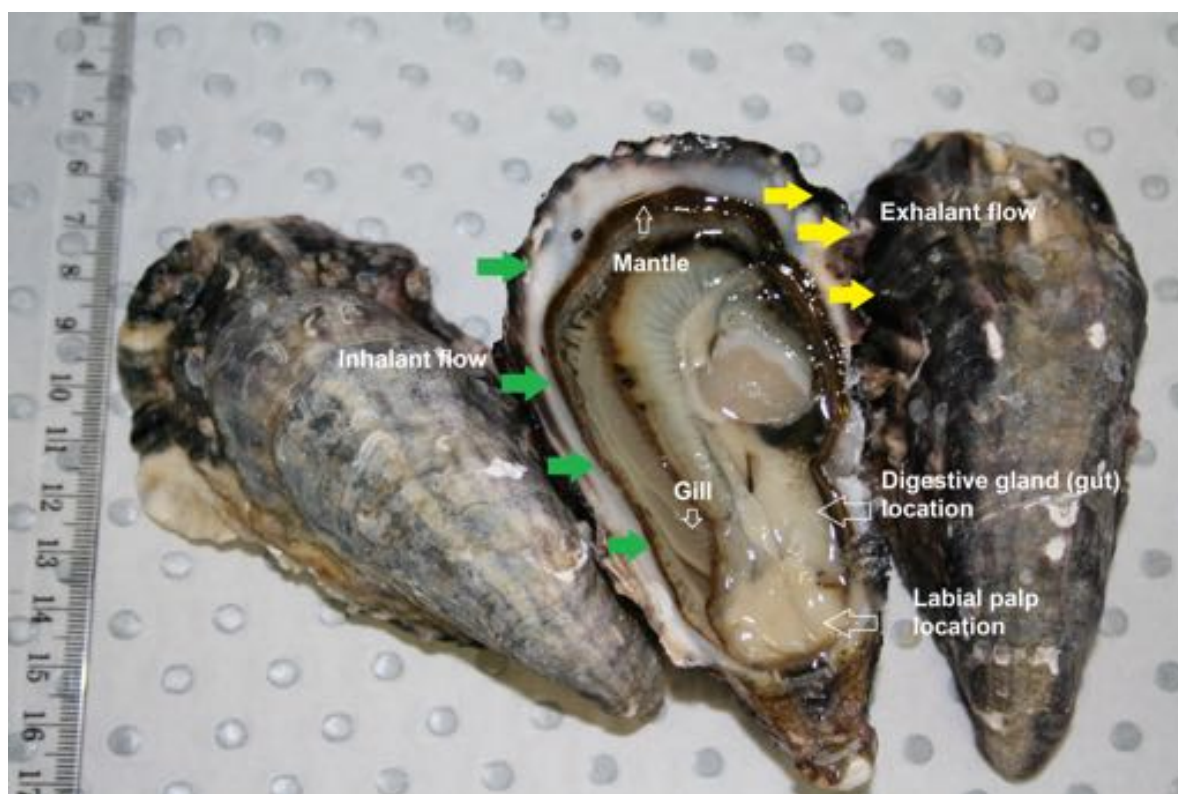
Not only is the impact of a foodborne outbreak a concern but transmission to the rest of the community is an additional burden. An outbreak of NoV illness linked to steamed oysters showed that secondary cases were observed in 20% of households and 14% of household members as a result of contact with primary cases (109). The reproduction number, which is the mean number of susceptible persons who become infected by an infectious individual, for NoV is likely to vary greatly (110) with 2 reported in hospital settings (111) and 14.05 observed at a world scout jamboree (112). As soon as contaminated oysters have infected a consumer with NoV, the disease may spread into the community. The 525 cases reported for the Tasmanian outbreak, which was linked to contaminated oysters were primary cases (97).

## 1.5 Oysters

### 1.5.1 Oyster biology

Oysters are bivalve molluscs and are filter feeders. In Australia the main species of farmed oysters are Pacific oysters, SRO and *Ostrea angasi* (native oyster). Ninety seven percent of Australian oysters are produced for the domestic market. In 2011-2012, 33% of oysters were produced in NSW, 41% in SA and 25% in Tasmania (113).

Oyster species can be differentiated by the appearance of their shells. The shell protects the soft bodied bivalve mollusc from predators and from desiccation when the oyster is out of the water. It also helps direct particles (food) across the gills by controlling the water flow through the oyster. Calcium carbonate is the main component of the shell and is taken up from the seawater (114).



**Figure 1.2:** Oysters, showing anatomical components of the Pacific oyster.

The two sides of the mantle, which encloses the animal inside the shell, consist of connective tissue with nerves, muscles and haemolymph vessels (Figure 1.2). The heart pumps haemolymph around the body of the oyster. The inner surface of the mantle is lined with cilia which direct the food particles aggregated in mucous onto the gills. The cilia also deflect heavier particles along rejection tracts towards the entry point on the mantle for the incoming water.

Periodically the oysters will “blow-out” the rejected material as pseudofaeces. In oysters, the labial palps and mantle are responsible for the rejection of pseudofaeces in the exhalant current. Pseudofaeces are loose and easily distinguished from the more compact faeces. In oysters, the surface area of the gills has been increased by plica or folding. Coarse filtration occurs through the plica and the folds filter finer particles. Gills also serve a respiratory role, enabling gas exchange with the haemolymph. They are also involved in the bioaccumulation of pesticides, soluble heavy metals, hydrocarbons and some viruses. Each gill finishes within a pair of triangular-shaped palps situated on each side of the mouth which remove material from food tracts on the gills (114) (*see* Figure 1.2).

Particles from the gill travel along acceptance tracts to the mouth. Ciliary movement helps to propel material from the mouth to the stomach. Digestion occurs in the stomach and the digestive gland. The waste products form faeces and are discharged through the anus. The oyster has a simple nervous system consisting of three pairs of ganglia and several pairs of nerves.

Oysters draw in seawater and filter out the nutrients (114). Pacific oysters can filter up to 2 -5 litres of water an hour (115). Oysters feed opportunistically and select quite carefully what they feed on, preferring nitrogen-rich nutrients (116). They can however, accumulate and concentrate pollutants in their normal processes of feeding. Some bacteria and viruses such as *E. coli* and poliovirus can be cleared out of the shellfish naturally in the growing water by tidal flushing with clean water or by the mechanical process of depuration, NoV cannot however, be removed by these processes (117).

### **1.5.2 NoV binding in oysters**

NoV can be detected in oysters within one hour of the virus polluting their growing water (118). NoV does not multiply in the oysters. Oysters continue accumulating NoV to concentrations higher than that in the surrounding water (118) and the virus remains in the oysters for up to two months after the initial contamination (119). Accumulation of NoV within oysters can depend on oyster species and NoV genotype (118, 120).

#### **1.5.2.1 Distribution of NoV in oyster tissues**

McLeod *et al.* (121) demonstrated that following 48h of bioaccumulation with NoV GII.4, Pacific oysters had higher concentrations of NoV RNA in the digestive tract than in the gills and labial tract. GII.4 interacts with oyster digestive tissues using both sialic acid in an  $\alpha$ 2,3 linkage and an A-like carbohydrate ligand (122). NoV immunoreactivity was indicated in the lumen and epithelium of the digestive tract tissue (stomach, intestine and digestive diverticula) and in connective tissue (122). It was further proposed by Maalouf *et al.* (118) that GII.4 is accumulated on sialic residues in the gills and mantle where the virus is destroyed, preventing GII.4 accumulation in the gut, whereas GII.3 accumulates in the gut, possibly *via* sialic acid ligands in the gills and mantle but with a lower binding affinity. NoV GI binds to strain-specific carbohydrate moieties within the oysters which are indistinguishable from HBGA A antigen (122, 123).

Most studies of NoV accumulation in oysters are conducted in the laboratory, as it is difficult to know beforehand, when a contamination event is to occur in shellfish growing water. Some studies have been performed using oysters placed in the path of a potential supply of NoV such as sewage treatment plant (STP) outfalls as most STPs do not remove all NoV during treatment (124, 125). For further information see S1.7.1.

#### **1.5.3 Detection of NoV in oysters**

A variety of methods have used to detect NoV in shellfish involving the recovery of virus, purification of viral RNA and then amplification of the genome (126-129). The difficulties have been to remove inhibitors of the amplification process found in the shellfish such as glycogen and to obtain enough clean viral RNA for the amplification to proceed. NoV is concentrated in the oyster digestive diverticula (gut) (121). The gut is dissected from the oyster, the surrounding white gonad tissue is removed and discarded, and the viral extraction is performed on the remaining gut tissue (Figure 1.3). Methods for virus recovery have included alkaline elution using glycine buffer (130), acid adsorption (131) and protease digestion in the virus recovery step (132). Some methods have also used polyethylene glycol to concentrate the virus (133). Following evaluation of these methods, standard methods ISO/TS 15216-1:2013 (quantification) and ISO/TS 15216-2:2013 (qualitative detection) were published in 2012 permitting comparison of results worldwide (134, 135). The standard method utilizes protease digestion for virus recovery followed by guanidine thiocyanate to disrupt the virus and then silica adsorption to

purify the RNA. The method uses real-time PCR to detect the viral genome, targeting the conserved region at the 5' end of ORF2.



**Figure 1.3:** Photograph showing a shucked oyster and a dissected oyster gut.

Costs for labour to prepare the gut and the molecular diagnostics to extract and amplify the NoV are high. Prior to the studies described in this Thesis, there were no laboratories with the capability of testing shellfish for NoV on a routine basis in Australia. Bentham *et al.*(136) in SA, used reproducible PCR methods for detection of HAV, NoV GII, Adenovirus and Astrovirus in oysters that had been bioaccumulated with the viruses in the early 2000's but the methods were not used routinely. Similarly SA Pathology, based in SA had trialled their in-house PCR method to detect NoV in oyster homogenate but did not use the method routinely (R. Ratcliff 2009 *pers. comm.*). Instead, Australian shellfish suspected of viral contamination were sent to New Zealand for NoV analysis, adding to the cost and time taken to obtain results.

### **1.5.3 Elimination of NoV from oysters**

NoV can remain in oysters situated in growing waters for up to 2 months after the contamination event. Dore *et al.* (137) tested naturally- contaminated Pacific oysters for NoV GII that were relayed into a clean growing area, for 17 days. Virus genome copies were reduced from 2900 to 492 per gram. Depuration for six days reduced the NoV GII further to below 100 genome copies per gram. Greening *et al.* (119) investigated the reduction of NoV in Pacific oysters, following

in-tank bioaccumulation of NoV GII.3 and subsequent placement of the oysters in the sea and found that the virus could be detected intermittently for up to eight weeks. A study following the elimination of NoV from Pacific oysters held in depuration tanks at 16°C over a period of two weeks, was conducted by Neish (138) who found a total reduction of 41% in genome copies per gram.

#### **1.5.4 Inactivation of NoV in oysters**

Cooking oysters at home or in a restaurant for consumers anticipating a fresh or lightly poached oyster is inadequate for inactivating NoV (109, 139, 140). The internal temperature of bivalve molluscs should be maintained at 90°C for at least 90 s to inactivate NoV (141).

Flannery *et al.* (142) suggested that cooking mussels contaminated with NoV at > 90° C for longer than 3 min would make the mussels safer for the consumer although acknowledging at the same time that it is impossible to confirm the reduction in infectivity without a volunteer study.

The efficacy of high-pressure processing for NoV inactivation in fresh oysters is currently being studied as a processing alternative. Volunteers consumed contaminated oysters that had been treated at 600 MP for 5 minutes at 6°C. The high pressure processing was effective at inactivating NoV, however the very high pressure required to inactivate NoV produces a whitened, firmer oyster which has a very different and unappealing palatability when compared to the fresh untreated oysters (143, 144).

### **1.6 Shellfish Quality Assurance**

In Australia, oysters are farmed according to procedures and protocols described in the ASQAP Manual in growing areas classified as per the procedures described in the Manual, based on the probability of faecal contamination of growing area (145). These areas are monitored for faecal coliforms (faecal indicator organisms) at a frequency prescribed by the requirements of their classification which was defined from information provided by a sanitary survey of the area prior to approval for oyster farming in that area.

ASQAP is modelled on the United States NSSP. ASQAP mandates the implementation of sanitary surveys (risk assessment) of production areas to identify potential sources of pollution and provisions for the testing of faecal bacteria, e.g. *E. coli* and faecal coliforms, in shellfish and their overlying waters to reduce the risk of human pathogens in shellfish. Regular monitoring of *E. coli* and/or faecal coliforms in oysters and growing waters is undertaken as part of the ASQAP



according to the sanitary survey classification. ASQAP has been adopted by each shellfish producing State and Territory of Australia and is overseen by representatives from State and Territory shellfish authorities, Industry, Food Standards Australia New Zealand and the former Australian Quarantine and Inspection Service, now part of various divisions of the Australian Department of Agriculture.

The three main oyster producing States in Australia, NSW, TAS and SA have Shellfish Quality Assurance Programs that are compulsory government and industry co-funded, programmes to manage the safety of oysters for the consumer. In NSW the programme is administered by the NSW Food Authority under the NSW Food Act 2010. In Tasmania, the programme is administered by TSQAP under the Tasmanian Public Health Act 1997. In SA the South Australian Shellfish Quality Assurance Program (SASQAP) administers the shellfish programme under the South Australian (Primary Produce) (Food Safety Scheme) Food Act 2004 and (Primary Produce) (Food Safety Scheme) Regulations 2006. Each programme adheres to the principles and objectives of the ASQAP.

### **1.6.1 Classification of harvest areas**

Shellfish harvest areas in Australia vary in their potential exposure to faecal pollution sources from ‘pristine’, meaning no human habitation, land-based farming or other pollution sources in the catchment (*e.g.*, such as in Coffin Bay, SA) to those exposed to large populations in the catchment, such as around Sydney, NSW. Harvest areas are inspected in accordance with ASQAP and a sanitary survey (risk assessment) defines the classification of the area (145). Classifications vary, accordingly, from “Approved” to “Conditionally Restricted” and, in areas with extreme potential exposure to faecal pollution risk, “Prohibited”. Harvest areas with conditional classifications are subject to closure in response to defined environmental conditions *i.e.* rainfall, sewage release *etc.*

Table 1.1 shows the number and type of harvest areas by classification in the three States. More Approved harvest areas are found in SA, reflecting the remote nature of most of the commercial shellfish-growing areas. In NSW there are more Conditionally Restricted harvest areas which reflects the much higher population density and higher risks for contamination of the growing areas.

In comparison, in 2013, UK had 355 beds of which 2 were class A<sup>1</sup>, 325 beds were Class B and 28 beds classified as C (147). Australian oyster harvest areas with Approved classifications are equivalent to UK class A.

**Table 1.1:** Number of harvest areas and classifications by production region, as at August 2014.

Harvest area classification	SA No. of harvest areas	TAS No. of harvest areas	NSW No. of harvest areas
Approved	24	8	
Approved conditional	4	35	32
Restricted conditional			45
Prohibited			2
Other			Inactive 2
	Temporarily approved 1		Not active 2
			Pending 6
			Unclassified1

A 2012 EFSA expert scientific opinion on NoV in shellfish (140) stated that ‘the most effective public health measure to control human NoV infection from oyster consumption is to produce

<sup>1</sup> UK classification of shellfish production areas.

- Class A ( $\leq 230$  *E. coli*/100g) - molluscs can be harvested for direct human consumption.
- Class B (90% of samples must be less than or equal to 4600 *E. coli*/100g) - molluscs can go for human consumption after purification in an approved plant or after relaying in an approved relaying area or after an EC approved heat treatment process. All samples must be less than 46000 *E. coli*/100g
- Class C ( $\leq 46000$  *E. coli*/100g) - molluscs can go for human consumption only after relaying for at least two months in an approved relaying area followed, where necessary, by treatment in a purification centre, or after an EC approved heat treatment process.

In all cases, the health standards in Annex III of European Regulation 853/2004 and the microbiological criteria adopted under European Regulation 2073/2005 must be met. 146. **Coates L.** 2015. Protocol for the Classification of Shellfish Harvesting Areas – England and Wales. CEFAS  
<https://www.cefes.co.uk/media/52553/201504-classification-protocol-revised-version-12-final.pdf>

oysters from areas which are not faecally contaminated, particularly given the ineffectiveness of current depuration and relaying procedures’.

## **1.6.2 Triggers for closure of harvest areas**

### ***1.6.2.1 Faecal indicators for detecting raw or partially treated sewage in harvest areas***

*E. coli* and faecal coliform levels are used as regulatory food safety criteria for management of the safety of oysters and their harvest areas in Australia. They are routinely excreted in human and animal faeces and are considered a useful microbiological indicator of faecal contamination in oysters and seawater immediately following faecal contamination events (148). However, levels of *E. coli* and coliforms in oysters and growing waters have been shown to be reduced within a few days due to elimination and inactivation under tidal and environmental influences (149-153), whereas the NoV genome has been detected in oysters intermittently for up to 2 months following contamination (119). It is not known how long NoV will remain in SRO, following a contamination event.

### ***1.6.2.2 Rainfall***

Agricultural runoff occurs when rainfall flows down through paddocks and buildings where animals have been kept, or through non-pastoral land and bush land where native or feral animals live. The runoff can carry animal faeces into water bodies that may potentially include oyster growing areas.

Runoff in populated areas can potentially include pollution from, for example: failing septic tanks/on-site sewage management systems (OSMS), cracked or broken sewage pipes and illegal connection of sewage pipes to stormwater systems. Flooding can also cause OSMS valves/ lids to burst open and introduce raw sewage into the floodwater.

Rainfall is monitored in each growing area according to the ASQAP sanitary survey. When the rainfall reaches a prescribed level or trigger, the growing area may be closed for harvesting due to potential release of pathogens from sediment particles as salinity decreases. River flow can also be monitored as part of the sanitary survey and used as a trigger to indicate potential faecal contamination from runoff.

### ***1.6.2.3 Salinity***

Salinity is also monitored in growing areas according to the sanitary survey (145). Changes in salinity can trigger closure of the growing area because salinity can be reduced following rainfall

and runoff into the growing area. The salinity of a growing area can increase in dry weather, when water inputs into the growing area are reduced. There is also a risk of contamination of the oysters because of reduced freshwater flushing.

#### **1.6.2.4 Sewage**

When shellfish authorities are notified of sewage overflows that will impact the harvest areas, the areas are closed for harvesting until they are cleared of NoV or faecal pollution and there is no further risk from the original pollution source. Of the last five NoV illness outbreaks traced back to oysters from Australian growing areas contaminated with untreated sewage, one area has been closed for direct harvesting for the past six years because of the many potential sources of contamination (154). The other two areas, contaminated by leaking sewerage pipes, were closed for three and eleven months respectively (97, 106). Investigations of each of the last five NoV outbreaks were conducted by the respective State Shellfish and Health authorities. Analyses of shellfish samples were conducted by ESR (NZ) and SARDI Food Safety (PIRSA SA) (97, 106, 154).

### **1.7 Contamination of growing areas with NoV**

Contamination of oysters with NoV in their growing areas occurs when untreated human excrement flows into the growing areas (155-158). The virus is taken up by oysters as part of their feeding process. NoV can survive for long periods in the environment, particularly in sediment (50). Contamination of shellfish with NoV has occurred from the effluent of STPs under normal operation (124, 159), pump station sewage overflows (160), shellfish workers (161, 162) and has been suspected to have arisen from caravan park sewage spray run-off, septic tank leachate (*see* Figure 1.3) and marine vehicle discharges (163).

In Australia, viral outbreaks have occurred following contamination of oyster growing areas from OSMS leachate; broken sewerage pipes, pump station overflows, houseboats, pit toilets and unsewered tourist parks (7, 11). Notably large outbreaks of viral illness occurred due to NoV contaminated oysters from the Georges River, NSW in 1978 (n>2000) and Tasmania in 2013 (n = 525) and from HAV linked to Wallis Lakes, NSW (n=444) in 1997 (97, 106, 164).



**Figure 1.3:** OSMS near to waterway and oyster harvest area

### 1.7.1 STP effluent in oyster growing areas

The presence of STPs with outflows potentially reaching Australian oyster growing areas is taken into consideration in the classification of the harvest area, with a “prohibited closure area” defined around the outflow assuming that the treatment process and the dilution of the effluent is enough to remove the risk of NoV contaminating the oysters. In the EU, as of 2011, there was no legislation to prevent harvesting in the immediate vicinity of sewage outflows. This is being remedied through the introduction of sanitary surveys to document potential pollution sources (89). Currently, production areas in which shellfish have *E. coli* > 46000 CFU/100g are not to be harvested for consumption and cannot be purified, see footnote p. 21).

Research has shown that during the normal operation of STPs, enteric viruses are not removed as effectively as bacterial pathogens and currently used bacterial indicators (124, 157). The capacity of the STP to reduce NoV during normal operation varies according to the type of treatment plant *e.g.* primary treatment plants *cf.* membrane bioreactor treatment plant; the load of virus entering the plant; plant residence times and flow rates. The concentration of NoV reduced from the influent varies from approximately  $1\log_{10}/L$  for a primary plant to  $6\log_{10}/L$  for a membrane bioreactor plant (165). Sima *et al.* (165) studied the reduction of NoV through a membrane bioreactor treatment plant and proposed that the virus attaches to particles in the aeration basin, are then retained by the membrane and removed as biosolids from the plant.

Eastern oysters located in Mobile Bay, Alabama, USA, 5.74 km away from the discharge point of a wastewater treatment plant and at dilutions of seawater to effluent of 158:1 to 556:1 were contaminated with NoV GII (159). Similarly, NoV was detected in mussels that were located 10 km away from a large primary sewage treatment plant (STP) outflow in New Zealand (166). Ueki *et al.* (157) detected NoV and sapovirus in oysters, approximately 1 km away from an STP outflow. Information about the distance that NoV can be dispersed and be taken up by oysters, and how long the virus will remain in oysters is limited. Uptake of NoV into oysters can vary according to species and NoV genotype (68, 118, 120). No studies have been conducted in Australian waters using SRO.

### **1.7.2 Post harvest depuration**

Commonly practiced regimes of depuration and relaying are used in Australia (and worldwide) to reduce the faecal contaminants in oysters. Depuration in NSW involves placing the oysters in clean water and allowing them to purge out the contaminants for 36h. Relaying is the removal of oysters from their growing area to a clean growing area for 14 days or longer, to permit purging contaminants (167). Neither method as currently used in the EU, effectively reduces NoV in oysters (140). This has also been observed in Australia where outbreaks of NoV gastroenteritis have occurred following the consumption of depurated oysters (103, 106, 154). Other alternative processes to inactivate NoV in oysters have been discussed in S1.5.4.

### **1.7.3 Alternative indicators of sewage contamination in oyster growing areas**

#### ***1.7.3.1 Bacteriophage testing***

F+ RNA bacteriophage testing is used by the FDA in conjunction with faecal coliform indicators as an additional indicator of untreated sewage contamination in shellfish growing areas. In cases of raw sewage discharge, the NSSP (168) prescribes that analytical results shall not exceed 50 male specific coliphage per 100 grams of shellfish tissue for samples collected no sooner than seven days following the contamination event (169). A variety of groups of these bacteriophage are found in the faeces of humans and animals. F+ RNA bacteriophage Group II and III are more prevalent in human faecal sewage whereas Groups I and IV are generally associated with animal faeces. If the bacteriophage are present in high enough numbers, the Groups can be differentiated using molecular techniques (170). In 1999, Grabow *et al.* (171) noted that bacteriophage are excreted by a certain percentage of humans and animals all the time, whereas viruses are excreted only by infected individuals for a short period.

The Institute of Environmental Science and Research (ESR) in New Zealand, has trialled the use of bacteriophage as an indicator of faecal contamination of shellfish. They stated ‘no clear predictive correlation between the occurrence of enteric viruses and F+ RNA bacteriophage was observed in shellfish impacted by faecal contamination except where shellfish were growing in close proximity to a sewage outfall’ (166). F+ RNA bacteriophage are susceptible to degradation due to sunlight and have been shown to lose viability rapidly in water after exposure to UV light at similar strengths to sunlight (172). It should be noted that the “summer - strength UV light” used by Flannery *et al.* (172) was the equivalent of winter sunlight found in NSW. A depuration study by Polo *et al.* (173) has shown that F+ RNA bacteriophage lose viability after being taken up by clams and mussels at a rate of approximately 1 log per week (clams) and 2 log per week (mussels) although the bacteriophage in the mussels were reduced to < 10/ 100 g shellfish which may imply almost total removal. Similarly, Neish (138) found that Pacific oysters showed a reduction of 1 log per week of F+ RNA bacteriophage over 14 days at 16°C. The impact of sunlight on phage and fluctuating levels of NoV within the New Zealand communities may explain the ESR results.

## **1.8 Prevalence of NoV in oyster growing areas**

Internationally, Suffredini *et al.* (174) reported that NoV GII.4 and GII.g were detected in 8.3% (n = 120) of Italian shellfish collected from category A and B production areas. Nishida *et al.* (175) detected NoV GI or GII in 9% of Japanese oysters (n = 191) collected from two different production areas. A 2011 study undertaken in the UK (176) notes that 76.1% (n = 844) of samples collected from oyster production areas gave positive results for NoV GI and or GII. NoV was detected in 63% of all Category A area samples, 78% of category B area samples and in 88% of the samples from Category C (176). In the USA 20% of oysters (n = 45) sampled from 33 bays in summer and 12 bays in winter and purchased from farmers, wholesalers and retailers were positive for NoV GII.4 (46). A separate US survey, however, demonstrated the presence of NoV in only 3.9% of oysters (n = 388) collected from retail outlets in 9 USA States (177) where 1% (4/388) were GI and 2.8% (11/388) were GII.

Direct comparisons of the occurrence of NoV in oysters produced in different countries are confounded by the use of different survey and sampling approaches (*e.g.* retail vs. production area) and analytical methodology. The UK survey of Lowther *et al.* (176) used ISO/TS 15216 (134) which incorporated a proteinase shellfish extract digestion and a silicate RNA purification

(128) for detecting NoV in shellfish and will allow future comparisons of results. Very little information exists about sampling regimes for testing oysters in growing areas for NoV.

At the commencement of this study there were no data on the prevalence of NoV in Australian oyster growing areas apart from epidemiological evidence of outbreaks linked to Australian oysters.

## **1.9 NoV illness outbreaks and Australian oysters**

As noted earlier, illness outbreaks due to NoV contamination in Australian oysters occur at a rate of approximately 1 to 2 a year (178). Over the last decade, the number of outbreaks due to contaminated oysters has decreased tenfold, with the decrease attributed to the introduction of an Australia wide quality assurance programme (13). There have been 18 illness outbreaks associated with Australian grown oysters over the past 11 years, including the large outbreak of illness in 2013 linked to oysters from Dunalley, Tasmania (97, 179).

Improvements in molecular testing of oysters have led to the identification of NoV as being the causal agent for each illness outbreak occurring during the last five years. In the previous five years, the causal agent was suspected to be NoV (but not detected) in 13 illness outbreaks (15).

### **1.10 Burden of NoV illness in Australia**

The economic burden to Australia of outbreaks of NoV illness associated with contaminated oysters was estimated by Hudson (180) as \$11.85 million/year. This was calculated using data from 2001-2010 based on the average occurrence of an oyster derived NoV event happening twice per year. It included the cost to the community in loss of health, welfare and life. Not included in this calculation was the cost to the oyster industry supply chain, in disruption costs and loss of profits which was estimated as several million dollars per NoV event.

Approximately 3 million dollars is spent on Australian shellfish safety annually. This includes the cost of testing for faecal bacteria and also the management of heavy metals and biotoxins (A. Zammit (NSWFA), C. Wilkinson (SASQAP) 2015 *pers. comm.*).



## 1.11 Summary

This review has identified gaps that relate to the purpose of this thesis:

- i) Australia does not have capacity for testing for NoV in shellfish on a routine basis
- ii) There is a lack of information worldwide about sampling regimes for the detection of NoV in shellfish
- iii) The prevalence of NoV in Australian oyster growing areas is unknown
- iv) There is minimal data available worldwide about the transportation of NoV in estuaries following sewage spills
- v) There is no data on the spatial and temporal distribution of NoV and uptake by SRO in an estuary following a sewage spill
- vi) There is little known about the risk of NoV illness to Australian oyster consumers

## **1.12 Thesis Objective**

### **1.12.1 Aim**

To establish an informed strategy for minimising the risk of NoV prevalence in Australian commercial oysters.

### **1.12.2 Objectives**

- i) To introduce a testing method for NoV in Australian oysters
- ii) To develop and trial a sampling programme for investigating NoV contamination in Australian oysters
- iii) To determine the prevalence of NoV in Australian oysters
- iv) To investigate the spatial and temporal spread of NoV in SRO following a sewage spill in a river estuary
- v) To evaluate the risk management of Australian oyster harvest areas for the protection of oyster consumers from NoV illness and provide strategies for minimising the risk to the consumers.

# Chapter Two: Methods

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## 2.1 Preface

This chapter presents experimental methods used throughout this Thesis, including methods for detecting NoV and HAV in oysters. Prior to this Thesis these methods had not been used in Australia to test Australian oysters. Validation of the NoV detection method in oysters was conducted for the routine use of the method in this laboratory and considered NoV strains and oyster species (including SRO) available commercially in Australia.

A preliminary investigation for the presence of human bocavirus (HBoV) in sewage-contaminated oysters is also described. That investigation, although a novel exploration, was considered not to be substantial enough to be a “stand-alone” chapter.

## 2.2 Introduction

### 2.2.1 Method for NoV detection in oysters

There have been many methods published for the extraction and detection of NoV in oysters (127, 181, 182), however, an international standard method (e.g. ISO or CEN standard) had not been published at the time of commencement of this Thesis. The method selected in this study for the extraction and detection of NoV in oysters published by Greening and Hewitt (129), is routinely used for the analysis of NoV in oysters in New Zealand. It has been shown to be both accurate and sensitive for detecting NoV in shellfish where the prevalence of NoV contamination is low, relative to that experienced in the EU. Therefore the method may be applicable for use in Australia, *i.e.* where epidemiological data suggests that NoV contamination of shellfish occurs infrequently. The method incorporates a proteinase shellfish extract digestion and a silicate RNA purification (128, 132) and is closely aligned with ISO/TS 15216 (134, 135), published in 2013, for detecting NoV and HAV in foods.

Particular concern for the sensitivity, accuracy and specificity of the NoV method was driven by the potential impact of detecting NoV in Australian oysters. A false negative sample could endanger the health of the consumer and a false positive sample could potentially close a harvest area for many years whilst investigators try to find the pollution source. Accordingly, validation

of the NoV detection method in oysters was conducted for use of the method in this laboratory and considered NoV strains and oyster species (*i.e.* SRO) found in Australia,

### **2.2.2 HAV detection**

There has been only one HAV outbreak linked to oysters (approx. 500 cases with one death) in Australia which occurred in 1997 (164). The last major foodborne HAV outbreak in Australia in which, imported semidried tomatoes were implicated (reported cases  $\geq 562$ ) in 2009 (183). The incubation period for HAV is long and can take 15 -50 days after ingestion, before illness develops. This makes it difficult to assign the contaminated food source. The illness can be mild or severe and require hospitalization. A study of the semidried tomato-implicated Australian outbreak of HAV showed that 45% of those who reported symptoms were hospitalized (14, 183). Kirk (81) observed that that outbreak "highlights that Australia is vulnerable to large and serious foodborne outbreaks of HAV".

Petrignani *et al.* (184) reported that in the Netherlands during 2008-2010, 4% of reported suspected cases of HAV were foodborne and that, following molecular typing, the cases linked to foodborne transmission increased to 16 %, suggesting that there are more foodborne cases than currently acknowledged.

The true occurrence of HAV in Australian sewage and sewage contaminated oysters is not known although epidemiological evidence suggests that it is very low (15). The population in the catchment selected for further study as part of this Thesis (*see* Chapter 5), had one of the lowest vaccination up-takes in Australia in 2003 (185) which may have influenced the prevalence of HAV illness in the area. The method used for testing shellfish for HAV was routinely used in this laboratory and enabled testing of oyster samples.

### **2.2.3 Bocavirus: a potential alternative indicator for NoV**

Faecal coliforms are currently used as faecal indicators in shellfish growing areas, however they are not always reliable indicators of the presence or absence of NoV (156, 186). F+ RNA bacteriophage is used by the USA FDA as an alternative indicator for sewage contamination within 7 days of the event. This indicator was trialled by ESR (NZ) however, no predictive correlation between bacteriophage and the presence of NoV was observed (187).

Human bocavirus (HBoV) may be an effective alternative indicator for the presence of NoV. It is a parvovirus and was first identified in paediatric respiratory secretions in 2005 (188). HBoV

can become systemic and serological diagnoses of the virus correlates with high viral load in the nasopharynx and viremia (189).

Three additional bocaviruses HBoV2, HBoV 3 and HBoV 4 were detected in human faecal samples with viral loads of  $10^3$ - $10^9$ /g faeces (190). HBoV2 and 3 have also been detected in untreated sewage samples collected from twelve cities in the USA (191).

In a study of 197 children with paediatric acute gastroenteritis, Arthur *et al.* (190) found that HBoV2 was the third most prevalent virus after rotavirus and astrovirus. HBoV2 was observed in 17.2% of children whereas NoV was detected in 13.4%. HBoV is a highly variant virus characterized by rapid evolution which has made primer design difficult (192).

*Parvoviridae* are small 18-26nm, nonenveloped, single negative DNA 2-6kb long and are extremely robust in the environment. Canine parvovirus survives in the environment for up to 5 months (193). There is no published data on the detection of HBoV in shellfish.

#### **2.2.4 Methods Evaluated**

This chapter presents the experimental methods for NoV detection and enumeration used throughout this Thesis. This chapter also presents methods, and their evaluation, for:

- i) Detection of HAV in Oysters
- ii) Evaluation of Method Proficiency
- iii) NoV Extraction from Sewage
- iv) Sequencing of NoV
- v) Detection of Bocavirus in Sewage Contaminated Pacific Oysters
- vi) Enumeration of *E. coli* in Oysters

For ease of presentation and communication of subsequent results, the evaluation and validation of novel methods are described in this chapter. Where established methods are used in subsequent chapters, they are also described in this chapter but without further evaluation or discussion.

## 2.3 Methods

### 2.3.1 NoV Detection and Enumeration

The method for recovery and detection of NoV in oysters closely followed the protocol of Greening and Hewitt (129), which was adapted from that of Jothikumar *et al.* (132). It involved dissection of the oyster gut, a virus recovery step, extraction of viral RNA using a commercial kit, a separate reverse transcription (RT) of viral RNA to cDNA and Taqman real-time PCR assays for NoV GI and GII.

#### 2.3.1.1 Sample transportation and storage in the laboratory

Oyster samples were dispatched to the laboratory in polystyrene boxes with wrapped chiller blocks to keep the contents cool. Oysters from NSW (up to 2000 km from this laboratory) and Tasmania (1500 km from this laboratory) were shipped by road then air. Two flights were required to deliver the Tasmanian oysters. SA oysters were couriered 300 km by air. Oysters arrived at the laboratory alive and within 24 h of harvesting. The temperature of the oysters on arrival varied from 4°C to 12°C. The recommended storage temperatures for SROs is 10 - 21°C which is slightly warmer than that recommended for Pacific oysters < 10°C (167). For the purpose of testing for NoV and *E. coli*, oysters were stored at 4°C prior to testing, which was conducted within 24 hours of receipt of the sample.

#### 2.3.1.2 Preparation of shellfish homogenate and viral RNA extraction

Six to ten whole oysters per sample were washed and shucked, providing 2-7 g of digestive gland. The rest of the oyster was discarded. The white gonad material was removed and discarded and the remaining digestive gland was dissected, chopped finely and mixed with an equal volume w/v of Tris buffer 100mM, EDTA 20mM pH 8.5. Proteinase K (Roche, Australia) was added to a final concentration of 200 µg/mL. The sample was adjusted to pH 8.3 and the tissue digested at 37°C for 1 h in a Ratek Orbital Mixer Incubator (Ratek, Australia) rotating at 300 rpm. A further digestion step was conducted at 65°C for 15 minutes with an inversion of the mixture every 5 minutes. Two centrifugation steps were performed; the first was for 3000 g for 10 minutes in a Scanspeed 1580R centrifuge (Scanlab, Denmark); the supernatant was then decanted into microfuge tubes and centrifuged at 10,000 g for 5 minutes in a Scanlab 1730R

centrifuge (Scanlaf). Two tubes of 500  $\mu$ L of resulting supernatant per sample were held at 4°C overnight for RNA extraction the next day and the remainder was stored at -80°C. RNA was extracted from 500  $\mu$ L supernatant (equivalent to 0.5 g tissue per sample) using the High Pure Viral Nucleic acid Kit (Roche, Molecular Diagnostics, Mannheim, Germany).

#### ***2.3.1.3 Detection of NoV: Reverse transcription and qPCR.***

For each sample, cDNA was reverse-transcribed from 5  $\mu$ L of both an undiluted and a 1:10 dilution of RNA extract using reverse primers COG 1R for NoV GI and COG 2R for NoV GII (194). The 10  $\mu$ L reaction mixture contained 100 units SuperScript III reverse transcriptase (Invitrogen, CA, USA), 10 units RNase inhibitor (RNaseOUT, Invitrogen), 100 nM each of reverse primer, 1 mM each of dATP, dCTP, dGTP and dTTP, first-strand RT buffer and 5  $\mu$ L RNA. Reverse transcription was performed in a 5345 cycler (Eppendorf, Hamburg, Germany) at 50°C for 30 min followed by 95°C for 4 min and held at 4°C until the qPCR was performed. The qPCR reactions contained 5  $\mu$ L cDNA, 1x Platinum Quantitative PCR SuperMix-UDG (Invitrogen), 0.4  $\mu$ M primers COG 1F and COG 1R for NoV GI, COG 2F and COG 2R for NoV GII, Taqman probes 0.3  $\mu$ M RING 1(a) and 0.2  $\mu$ M RING 1(b) for NoV GI, and 0.2  $\mu$ M RING 2 for NoV GII (194) in a final volume of 25  $\mu$ L.

To July 2010, the qPCR assays for oysters sampled for Chapters 2, 3 and 4 were run on a Rotor-Gene 3000 and thereafter on a Rotor-Gene Q (Qiagen). Using either instrument, the temperature and time parameters were: 95°C for 5 min and 45 cycles of 95°C for 20 seconds followed by 56°C for 1 minute (156). The threshold was manually adjusted to 0.10 for all assays. The equivalent quantity of shellfish gut per qPCR reaction tube was 0.025 g.

#### ***2.3.1.4 Analytical controls***

A number of controls were included in each assay. Inhibition of the reverse transcription and qPCR by the oyster matrix was monitored by addition of Armored Norwalk Virus GI RNA (aIC, Asuragen, Texas, USA) prior to extraction, to 500  $\mu$ L of sample homogenate and to 500  $\mu$ L of water. Two replicate samples of the cDNA containing the aIC were assayed in a multiplex qPCR for NoV GI and GII and the aIC. The assay, primers and probe used for detecting the aIC have been previously described (129) and were included to indicate qPCR inhibition. aIC (GI) targets a section of the GI RdRp upstream, (towards the 5' end) and does not include RdRp sequences that are targeted by the NoV GI COG primers.

RNA extraction controls of a mixed NoV GI/GII faecal suspension (FC) and of water were included in each batch of extractions undertaken. NoV GI.3 faecal suspension was provided by M.Lyons (Queensland Department of Health, Australia) and the NoV GII faecal suspension was provided by R. Ratcliffe (SA Pathology, Adelaide, South Australia).

Serial ten-fold dilutions of “low”, “medium” and “high” levels of NoV GI and GII RNA were used as standards for the reverse transcription, qPCR and as standards to be compared with oyster sample  $C_T$  values. The NoV GII RNA standard was an RNA plasmid provided by R. Ratcliffe (SA Pathology) containing RNA of the C region of a strain of NoV GII.4. It had been prepared using the TOPO TA cloning kit (Invitrogen). SP6 was used to express the cleaned RNA and the DNA was removed using Turbo DNase (Invitrogen). The plasmid was quantified by optical density at 260 nm. qPCR RNA standard tubes containing “high” (6,600 copies), “medium” (660 copies) and “low” (66 copies) concentrations and a “no template” control (‘NTC’) containing water only were included in each GII qPCR. The concentrations of plasmid were selected to reflect potentially low concentrations of NoV in contaminated Australian oysters *i.e.*  $C_{TS}$  higher than 32.

The NoV GI RNA standards were prepared from a NoV GI.3 faecal sample described above, by extracting 500  $\mu$ L of a 1:500 dilution (in PBS) using the High Pure Viral Nucleic acid Kit (Roche). The GI RNA standards were serially diluted in 100 ng/mL PolyA (Roche) and aliquots stored at  $-80^{\circ}\text{C}$ . The concentration of the “low” GI standard was such that it would consistently be detected in the qPCR.

To monitor and to assess the repeatability of the assay,  $C_T$  results obtained for “high”, “medium” and “low” level NoV GI RNA, GII RNA plasmid standards and FC were recorded on a run-to-run basis. This enabled discrepancies to be detected and corrective action to be taken. The coefficient of variation (CV) was calculated for each RNA standard to allow comparison of individual distributions that differ in size.

To assess whether the method was capable of detecting the variety of NoV strains circulating in the community that may potentially contaminate Australian shellfish, a panel of faecal samples known to contain different NoV GI (GI.3) and NoV GII (GII.2, GII.3, GII.6, GII.6var, GII.12 and GII.14) strains obtained from clinical diagnostic laboratories in Australia (SA Pathology and QLD Health) was assembled. The laboratories had confirmed the genotypes by sequencing. The faecal samples were serially diluted, extracted and tested.



To explore the sensitivity and linear range of the assays, the RNA controls were serially diluted and amplified by real-time PCR to determine the end point dilution.

Experiments, described below, were undertaken to evaluate whether NoV GI and GII were able to be extracted from Pacific oysters and SRO using the chosen methodology. Percentage recovery rates for the extraction method were not able to be calculated as there was no external measure available to quantify the total amount of viruses that oysters take up from the water. Therefore, it was not possible to calculate the percentage of virus that the method extracted from the oysters. ISO/TS 15216 incorporates the use of mengo virus, a murine virus from the *Picornaviridae* family, as a process control. At the time of experimental set up for this Thesis, mengo virus was not permitted to be imported into Australia and no other alternative was immediately available.

#### **2.3.1.5 Spiking Experiment**

A preliminary experiment was undertaken which involved spiking a single sample of oyster gut homogenate with a faecal suspension known to contain NoV GI and NoV GII prior to the proteinase K digestion step. This was undertaken to assess whether the method could recover NoV from oyster gut tissue and that the tissue matrix did not cause significant inhibition of NoV amplification. The oysters were purchased locally from a retail fish shop. The faecal suspension was diluted 1:8 into the oyster gut, whereas the faecal suspension was undiluted.

#### **2.3.1.6 Bioaccumulation experiments**

The most appropriate positive sample for use in evaluating whether methods can effectively extract (recover) viruses from oysters are oysters known to be naturally contaminated with NoV as they are the same as what will be tested. At the commencement of this Thesis, no naturally contaminated Australian oysters were available for testing. Previous bioaccumulation studies have shown that live oysters can accumulate a variety of viruses in tank-based experiments (117). To further evaluate the recovery aspect of the method two small-scale bioaccumulation experiments were undertaken:

- thirty live Pacific oysters were exposed to raw sewage (influent) obtained from the Port Lincoln STP.
- ten live SRO were exposed to raw sewage (influent) obtained from the Urunga STP.

Thirty live Pacific oysters were placed in 1m x 1m tank in seawater to just cover them (100L). Sewage influent was trickled into the tank slowly with seawater until the water was cloudy. The

sewage and seawater taps were turned off and the oysters allowed to filter the water until it was clear. Fresh seawater was put into the tank until the oysters were open and feeding again. This was repeated five times. The tank was aerated for the experiment. Approximately 100L of effluent was used. The tank and water were decontaminated with bleach after use.

The bioaccumulation experiment using SRO was conducted in NSW. Influent was sourced from the STP at various times throughout the day to ensure a wide range of potential viral sources. Ten SRO were placed in a mesh basket in a 100L container with 80L of seawater. Influent was added at a rate of 1L per five minutes to a total of 20L. An aerator was used to aerate the water and disperse the influent. The oysters were open and filtering for 2 hours after the final addition of influent.

The oysters were then processed using the NoV and RNA extraction method detailed previously, and tested for NoV (GI and GII) by qPCR. This was undertaken to confirm that NoV could be recovered from oysters that had accumulated viruses in a more natural manner than can be simulated *via* spiking experiments.

#### ***2.3.1.7 Analysis of Naturally Contaminated Oysters***

Oyster samples found to be positive for NoV GI and NoV GII by ESR Ltd. (New Zealand) were imported and tested. Two samples of oysters were taken from the one pack (Korean Oyster A and B) to assess whether the method could recover and detect NoV in naturally contaminated oysters. The oysters were originally imported to New Zealand from Korea for human consumption and were linked to multiple human illness outbreaks of gastroenteritis in New Zealand. Armored RNA had not yet arrived in the laboratory and was not included in the NoV testing. Gel electrophoresis was undertaken on the qPCR amplification products obtained from the NoV GI and GII assays to confirm the product size.

#### **2.3.2 Detection of HAV in oysters**

HAV PCR analysis was conducted using extracted RNA that had been stored at - 80°C. RNA extracts, including undiluted, 1/10 and 1/100 dilutions were tested using a one-step PCR method as described in ISO TS15216-1 with an RNA UltraSense Kit (Invitrogen, Life Technologies)(134). Primers and probes for HAV, and reagents, and RT-qPCR cycling conditions were identical to those described in ISO TS15216-1. HAV positive RNA was serially diluted after extraction from plasma (provided by the National Reference Laboratory, Victoria, Australia) and was used as the “high”, “medium” and “low” positive RNA controls, at dilutions

of  $10^3$ ,  $10^4$  and  $10^5$  respectively (134). The HAV concentration of the plasma was estimated at  $10^7$  copies/mL using a Roche Lightcycler HAV Quantification Kit (Roche) by the provider. Certified reference material (CRM) for HAV (CEFAS, UK) was extracted as specified by the provider and included in the RT-qPCR analysis at an estimated concentration of  $5.5 \times 10^3 - 8.5 \times 10^4$  copies per reaction.

### **2.3.3 International Ring Trial/ proficiency testing**

The candidate participated in the European Union Reference Laboratory (EURL) NoV and hepatitis A virus proficiency testing scheme as part of the validation of the NoV detection method.

### **2.3.4 Sewage: sample preparation and RNA extraction**

Sewage samples were transported to the laboratory on ice and immediately frozen to  $-20^\circ\text{C}$  on arrival. The method of da Silva *et al.* (104) used for the extraction of NoV from sewage influent and effluent was as described in da Silva *et.al.* (104) with the following modifications. Briefly, viruses were concentrated from 80 mL influent/ effluent into 500  $\mu\text{L}$  by mixing 2 x 40 mL of sample with 10 mL of 50% polyethylene glycol 8000 (BDH, UK), 0.3M NaCl (Sigma Aldrich, USA). They were rocked gently overnight at  $4^\circ\text{C}$ . The mixture was centrifuged at 13,000g for 90 minutes at  $4^\circ\text{C}$ . The pellets were resuspended in 500  $\mu\text{L}$  of sterile distilled water and viral nucleic acids extracted and detected as described for the oyster samples (S2.3.1.2 and S2.3.1.3).

For both oysters and sewage, different steps of the overall analytical procedure (e.g. viral extraction, RNA extraction, reverse transcription and qPCR) were undertaken in separate workspaces to avoid cross contamination.

### **2.3.5 Virus sequencing**

Virus genome fragments detected in oyster samples and sewage were further characterized by cloning and sequencing. To confirm that the method was effective for NoV, human faecal samples derived from the panel (S2.3.1.4) and containing concentrations of NoV RNA that yielded  $C_T$  values of 30 - 34 in the qPCR, were cloned and sequenced first.

RNA was transcribed and amplified by PCR followed by a semi-nested PCR targeting the ORF1/ORF2 junction of the NoV genome. Transcription of the RNA used the primer G2SKR for

NoV GII and the assay mix described above (194). Each PCR reaction mixture of 25  $\mu\text{L}$  contained 0.8  $\mu\text{mol L}^{-1}$  primers, 0.5  $\text{mmol L}^{-1}$   $\text{MgCl}_2$  and 1  $\mu\text{L}$  Taq PCR Master Mix (Qiagen) and was performed in a Maxygene Gradient Thermocycler 1000 (Axygen, Calif. USA). For the PCR, G2SKR, Mon 431 (195) and 5  $\mu\text{L}$  cDNA were used, and for the semi-nested PCR, primers G2SKR and G2SKF (194) were used with 1  $\mu\text{L}$  of PCR product. The temperature and time parameters for the first round of PCR were 94 °C for 2 min, followed by 40 cycles of 94 °C for 30 s, 50 °C for 40 s, 72 °C for 60 s, followed by 72 °C for 10 min and for the semi-nested PCR were 94 °C for 2 min, followed by 40 cycles of 94 °C for 30 s, 50 °C for 40 s, 72 °C for 60 s, followed by 72 °C for 20 min. The PCR product was cleaned prior to the semi-nested PCR using a PureLink PCR purification kit (Invitrogen).

The final product was cloned directly into TOPO DH $\alpha$  *E. coli* cells and sequenced as described in the TOPO TA kit for sequencing (Invitrogen). Forward and reverse DNA sequencing was carried out using Big Dye terminator (BDT) chemistry version 3.1 (Applied Biosystems Corp.CA) and an Applied Biosystems capillary sequencer 3730-1 (Applied Biosystems) at the Australian Genome Research Facility Ltd. (Adelaide, Australia). DNA sequences were assembled using Vector NTi Advance 11.5 (Invitrogen).

#### **2.3.5.1 Sequencing and primer bias**

To investigate PCR and sequencing primer bias of primers used for sequencing NoV contaminated oysters as described in S2.3.5, a mixture of 7 GII strains (GII.2, GII.3, GII.4, GII.6, GII.6var, GII.12, and GII.14) and a GI.3 strain of NoV using faecal sample strains at approximately a  $C_T$  of 30 were prepared. Two mixtures, one of NoV GII.2  $C_T$  30 and GII.3  $C_T$  33, and one of NoV GII.3  $C_T$  30 and NoV GII.4  $C_T$  29 were also prepared for cloning and sequencing. Direct sequencing of the nested PCR product was also performed.

#### **2.3.6 Methods for the detection of HBoV in SRO and sewage**

DNA from oyster samples and sewage influent and effluent samples prepared for the NoV method were used. The extraction kit, High Pure Viral Nucleic acid Kit (Roche) was capable of preparing RNA and DNA from samples providing DNA for the amplification. Primers, IF, IIRa, IIRb (set A, HBoV 2) and IF, OR2 (set C HBoV 3) were provided by J. Arthur (SA Pathology, Adelaide) (190). Primers for set A and C were used in separate assays. The PCR mix is described in Arthur *et al.* (190). After activation at 94°C for 10 min followed by 60 cycles of amplification (94°C for 30 s, 52°C for 30 s, 72°C for 1.5 min) were performed.

Amplification products were visualized by gel electrophoresis in 2% agarose using TBE buffer and compared with faecal control HBoV 2 W153 products (provided by J. Arthur, SA Pathology, Adelaide) of approximately 230 bp (set A) and 448 bp (set C) primers (190). A 100bp ladder (Hyperladder IV, Bioline) was included in each gel to estimate the size of the fragments. The faecal control W153 diluted 1/100,000 was added to oyster sample DNA extracts to test for inhibition. A 1/10 dilution of the oyster sample DNA extract partially inhibited the assay, however a band of 230 bp was visualized in the gel. PCR assays were conducted with 1/10 and 1/100 dilutions of oyster and sewage DNA extracts.

The 230bp fragment was cut out from the gel and eluted using a kit, (Pure Link Quick Gel Extraction Kit, Life Technologies). The fragment was sequenced using the HBoV primer set A and the method described in section 2.3.5.

### **2.3.7 Enumeration of *E. coli* in oysters**

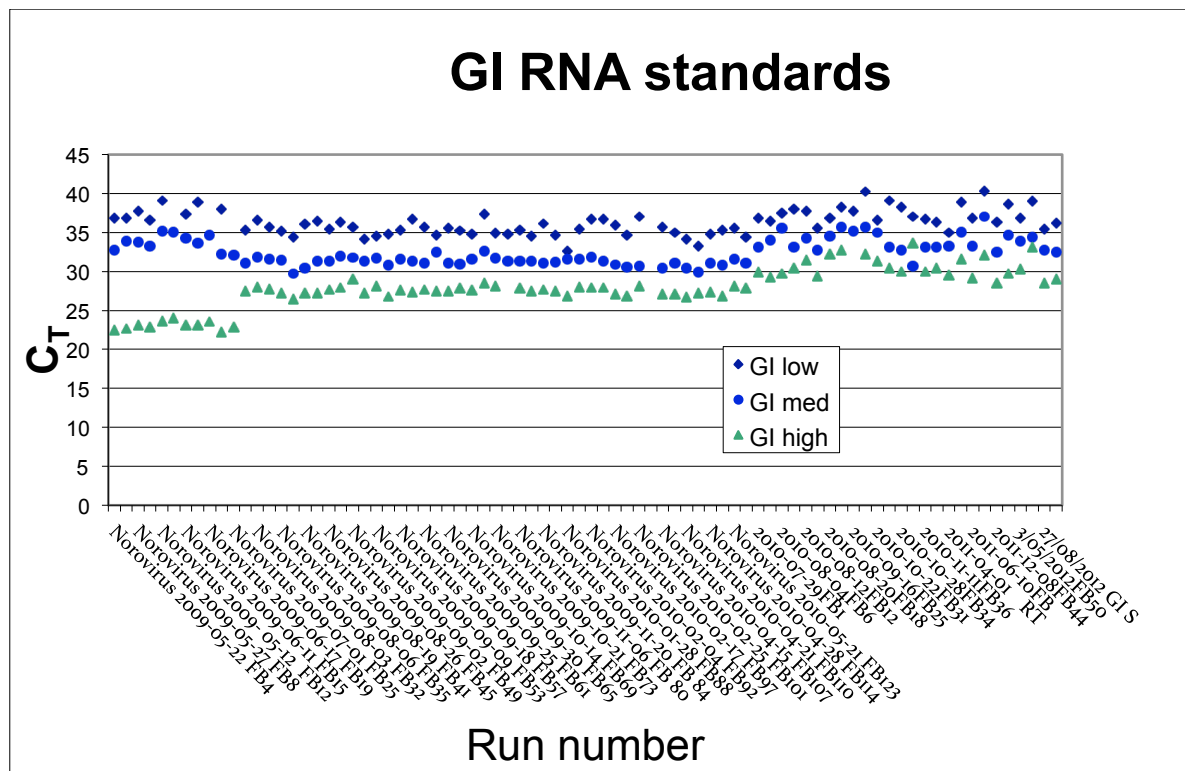
For each oyster sample, homogenates were prepared from the flesh and intravalvular fluid of six oysters and assayed for *E. coli* using the methods described in ISO/TS16649-3:2005 (E) (196) and Donovan *et al.* (1995) (197). Briefly, the method is a two-stage, five-tube, three-dilution most probable number (MPN) technique.

## **2.4 Results**

### **2.4.1 NoV testing method in Australian oysters**

#### **2.4.1.1 Control data**

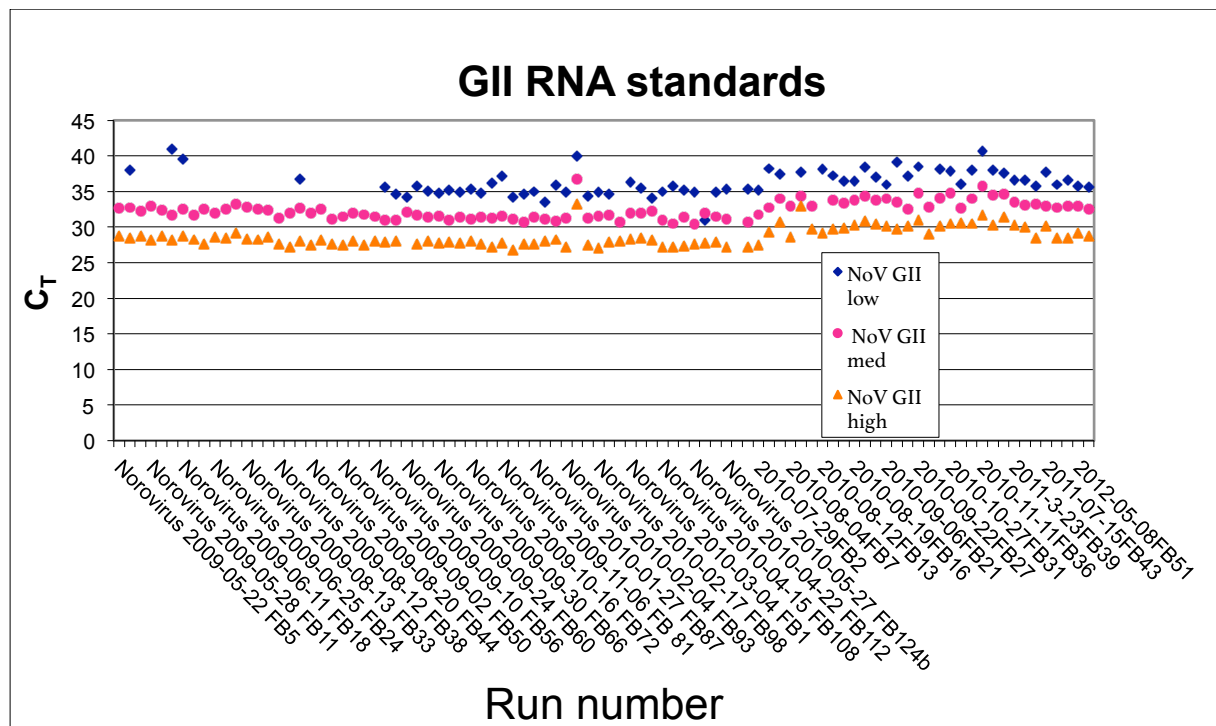
Eighty-five separate qPCR runs (batches of samples) were performed for NoV GI and 89 for NoV GII in our laboratory during the survey period. This included the survey samples, samples tested as part of inter-laboratory trials (198) for the studies described in subsequent chapters. The CV was calculated for the RNA standards and was less than 3.6% for NoV GI and less than 3.2% for NoV GII (Figures 2.1 and 2.1). Of the qPCR assays performed for NoV GI and GII for this study, all provided valid results for the qPCR standards. One assay had an  $R^2 = 0.983$ , the remaining twelve all had  $R^2$  values of  $> 0.992$ .



**Figure 2.1:**  $C_T$  of NoV GI RNA standards for each qPCR run. Runs conducted after 29<sup>th</sup> July 2010 were performed in a Rotorgene Q (Qiagen). A new set of NoV GI standards were prepared and used from run FB 27.

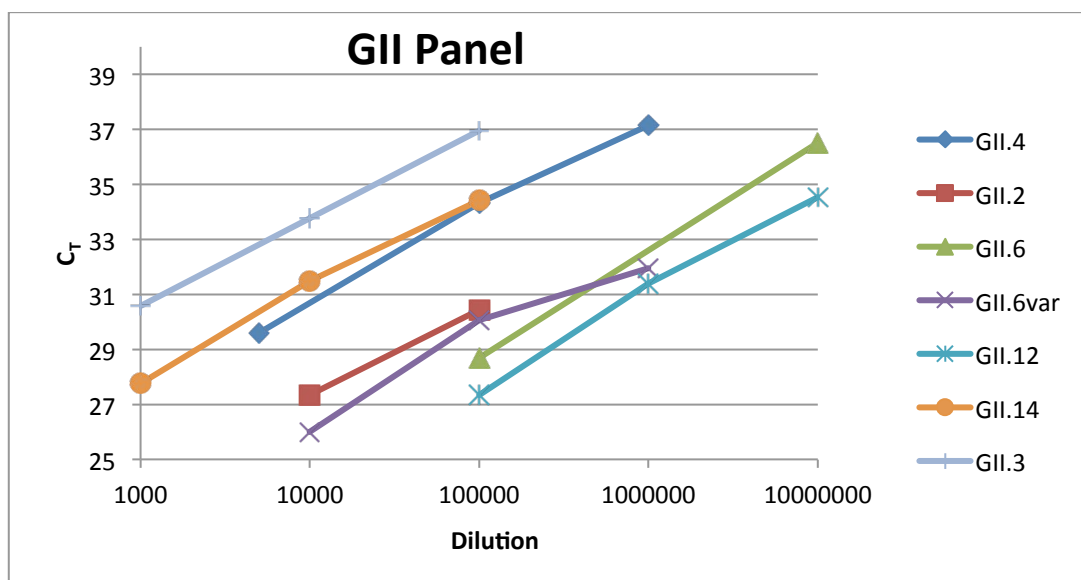
Runs conducted after 29<sup>th</sup> July 2010 were performed in a rotorgene Q (Qiagen). The new set of NoV GI standards were prepared and used from run number FB 27, which may explain the slight shift in  $C_T$  values for standards after this run.

Results for standards were recorded into charts (Figures 2.1, 2.2) three months after starting the study to indicate problems with the testing process. This drew attention to the sporadic results for the low NoV GII standard, which upon investigation revealed accidental freeze/ thawing of this standard. As a result, the NoV GII low standard was replaced and used from run number FB 56b onwards. All other controls had amplified reliably and generated consistently similar values during this time (Figure 2.2).



**Figure 2.2:** C<sub>T</sub> of NoV GII RNA standards for each qPCR run. Runs conducted after 29<sup>th</sup> July 2010 were performed in a Rotorgene Q (Qiagen). A new “low” standard was prepared from run FB 56b onwards.

A panel of known NoV positive faecal samples containing NoV GII genotypes and NoV GI.3 were analyzed to ensure that the methodology was capable of detecting the most prevalent NoV genotypes circulating in the community. The qPCR method for GI and GII was able to detect all the genotypes tested, GI.3, GII.2, GII.3, GII.4, GII.6, GII.6var, GII.12 and GII.14 (Figure 2.3).



**Figure 2.3:** qPCR results for known NoV GII positive faecal samples showing  $C_T$  vs dilution of faecal samples. Three serial dilutions of each sample were tested.

#### 2.4.1.2 Reliable Limit of Detection (RLOD) and Limit of Quantitation (LOQ) calculations

The Reliable Limit of Detection (RLOD) for NoV GI was estimated through endpoint dilution of the NoV GI RNA standards. The highest dilution of standard that reliably gave a positive result by qPCR (in 21 out of 24 tubes) produced an average  $C_T$  of 38.7 and was designated as one qPCR unit (qPCRu) and the (arbitrary) RLOD (*see* S.A.5). The limit of quantification (LOQ), was selected as the concentration tenfold higher than the RLOD (*e.g.* contained 10 qPCRu ml<sup>-1</sup> and was repeatably detectable) and was routinely used as the “low” RNA standard in each assay to ensure that each assay achieved appropriate sensitivity. The extreme endpoint of the assay *i.e.* the lowest concentration of GI RNA standard at which detection was possible was not determined.

The NoV GI titre of the samples was estimated through endpoint dilution of the NoV GI RNA standards and comparison of sample and standard  $C_T$  values within each run. No plasmid for GI was available during the NoV testing to enable quantification of the qPCRu in genome copies. Results from the Ring Trial proficiency testing, however, indicated that  $C_T$  values obtained for the proficiency trial were comparable and acceptable (198).

The RLOD for NoV GII was calculated by endpoint dilution of the RNA plasmid. The lowest concentration (6.6 genome copies) was detected in five out of 30 tubes and produced an average  $C_T$  of 40.4. Lower concentrations were not tested although it could be anticipated that detections at concentrations lower than 6.6 genome copies would be observed on occasion. All results



below the RLOD, with acceptable amplification curves were considered detects but were not quantified. The LOQ, designed to be repeatable to ensure consistency of detection, was selected as 66.6 genome copies (ten-fold higher than the RLOD) and was routinely used in each GII assay as the “low” RNA standard and amplified as indicated in Figure 2.2.

#### 2.4.1.3 Recovery

Both NoV genogroups were able to be detected when a faecal suspension containing a mixture of NoV GI and GII was spiked into an oyster gut sample (Table 2.1). NoV GI and GII were detected in the Pacific oysters that had been exposed to raw sewage influent from the Port Lincoln STP. SRO exposed to influent from the Urunga STP (Kalang River harvest area) were also positive for NoV GII (Table 2.1).

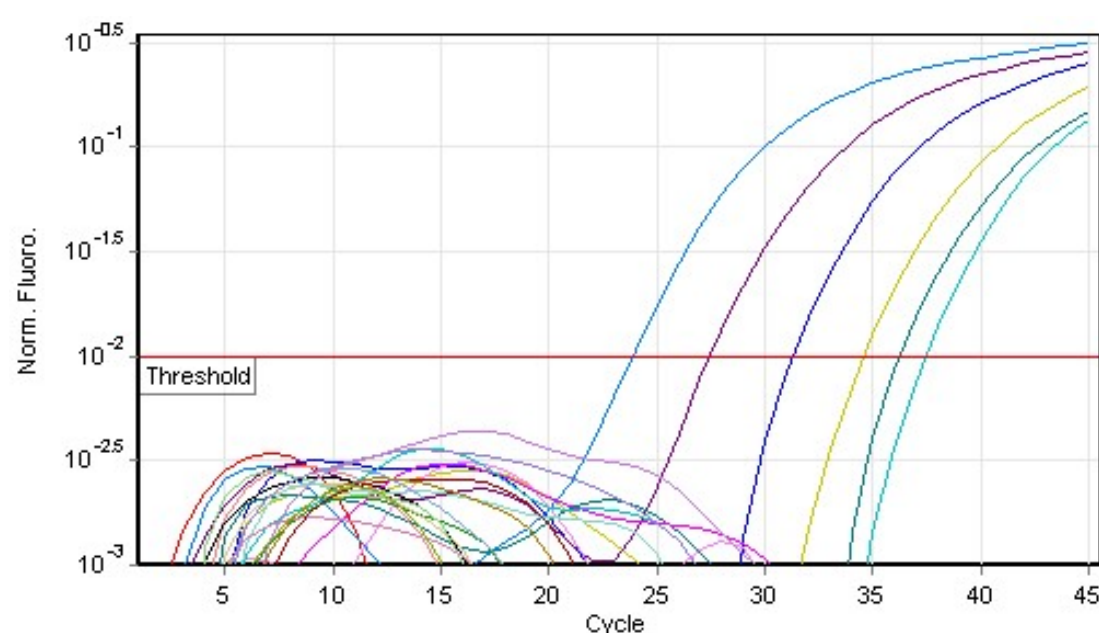
**Table 2.1:** Detection of NoV in oyster and faecal samples with different levels of contamination

Sample	NoV GI (C <sub>T</sub> )	NoV GI qPCR/ g shellfish gut	NoV GII (C <sub>T</sub> )	NoVGII copies/g shellfish gut
Oyster gut spiked with Faecal suspension	29.9	20520	34.5	4080
Faecal suspension	28.5	19000	32.5	17560
Pacific Oysters exposed to sewage influent*	40.1	Detect $\leq$ 40	32.1	18720
SRO exposed to sewage influent	$\leq$ RLOD	$\leq$ 40	35.9	1040
Korean oysters A <sup>#</sup>	36.3	1600	34.5	2710
Korean oysters B <sup>#</sup>	$\leq$ RLOD	$\leq$ 40	$\leq$ RLOD	$\leq$ 260

\* Oysters were found to have MPN of 24,000 E. coli/ 100 g shellfish,<sup>#</sup> Armored RNA was not included in NoV tests

#### 2.4.1.4 Analysis of Naturally Contaminated Oysters

NoV GI and GII were detected in one replicate sample of contaminated Korean oysters imported from New Zealand (Table 2.1 and Figure 2.4). The lack of armored RNA at the time of testing meant that inhibition of the qPCR was not tested for, and may have influenced the result, *i.e.* the concentration of NoV may have been higher than reported. The size of the qPCR products was confirmed using gel electrophoresis (not shown). Bands of 125 bp present in the qPCR product were thought to be long non-specific bands and bands smaller than 84 bp were considered primer dimers.



**Figure 2.4:** qPCR amplification profiles for NoV GI. Positive results were recorded and represent from left to right, FC, GI H, GI M and GI L NoV GI RNA standards, undiluted Korean oyster sample (KO) and 1:10 KO

#### 2.4.2 HAV detection in oysters

The results and discussion of the HAV testing are presented in Chapter 5.

#### 2.4.3 Sewage and virus detection

The results of testing sewage for NoV are presented in Chapter 5. The presence of HBoV in sewage is described below (S.2.4.7).

## **2.4.4 Virus sequencing**

### **2.4.4.1 NoV**

Human faecal samples used in the panel (S2.3.1.4) GI.3, GII.3, GII.4 were cloned and sequenced. The sequences matched their respective international reference genotypes. The results of the sequencing of NoV positive samples are presented in Chapters 4 and 5.

### **2.4.4.2 HBoV**

The results of sequencing PCR amplified product are presented in S.2.4.7 below.

### **2.4.4.3 Cloning and sequencing bias**

Direct sequencing of semi-nested PCR product detected GII.4 in the GII.4/ GII.2 mixture and GII.4 in the GII.3/GII.4 mixture. The sequence chromatograms showed some evidence of multiple strains in the mixtures but not enough to influence the result. The chromatogram of the GII.2/GII.3 mixture where GII.3 was tenfold less concentrated than GII.2 was unable to be read because of multiple sequences being present. Forty out of forty clones prepared (on several occasions, totalling forty clones) from a mixture of seven GII strains and one GI strain were sequenced and found to be GII.4. Ten out of ten clones prepared from the mixtures of GII.2 ( $C_T$  30) and GII.3 ( $C_T$  30) were sequenced and found to be GII.3.

## **2.4.5 International Ring Trial/ proficiency testing**

All viral and *E. coli* analyses conducted in this laboratory produced “acceptable” results in European Union Reference Laboratory Ring Trials for i) *E. coli* and Salmonella species trial 37 (199); ii) for NoV, trial 39 (198) and for NoV and HAV, trial 43 (200).

## **2.4.6 Enumeration of *E. coli* in oysters**

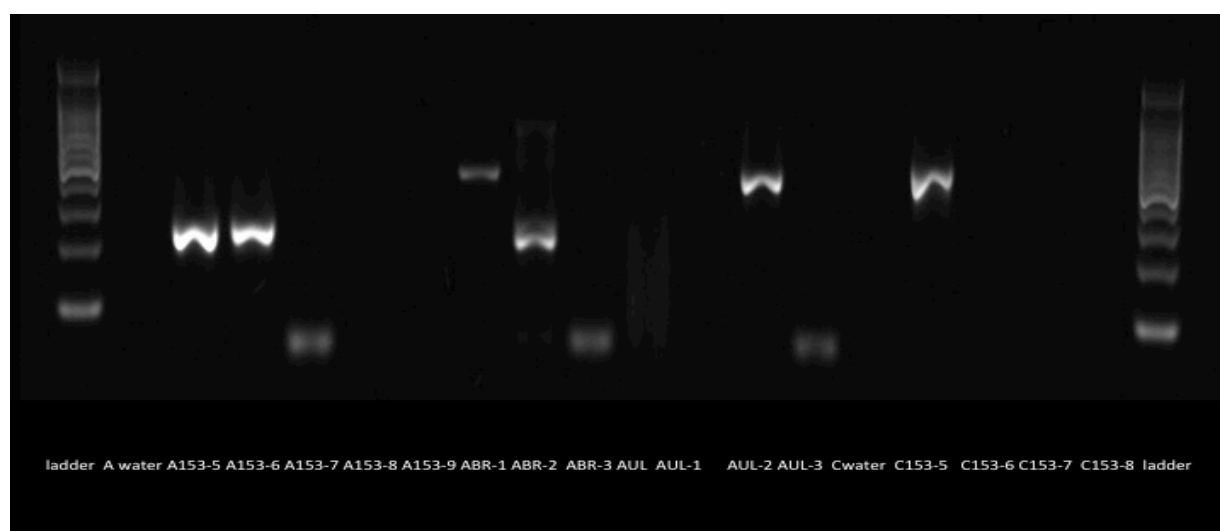
Results reported as MPN *E. coli* per 100 g of shellfish flesh and intravalvular fluid and are presented in S.3.4.5, S.4.4.2 and S.5.4.8.

## **2.4.7 HBoV detection in oysters and sewage**

PCR product of HBoV 2 and 3 was not detected in extracts of oysters contaminated with sewage collected as described in S.5.3, for

- day 5 site 1 and day 13, site 1 (NoV positive),
- BR a NoV positive oyster sample S5.3 and UL collected from lagoon near STP outfall S.3.2.1 and
- sewage influent and effluent DNA extracts S5.3.2.

A band of approximately 430 bp (C primers) was evident in the gel for the oyster samples from site 1 day 5 and day 13 and a band of 215 bp (A primers) as shown in Figure 2.5 A BR-3, but on sequencing, were determined not to be HBoV.



**Figure 2.5:** Photograph of a gel showing A and C primer, PCR products of 153 (HBoV2) dilutions  $10^5$  to  $10^9$ ; BR and UL oyster extract dilutions to  $10^3$ . Ladder in 100bp gradations.

## 2.5 Discussion

### 2.5.1 NoV testing method validation

The method of Greening and Hewitt (129) was selected for use in Australia because it was reported to be an accurate and sensitive method for detecting NoV in oysters. In this study, the method proved to be repeatable and capable of detecting NoV in Pacific oysters, SRO and sewage and detection of a range of NoV strains most likely to infect Australians, NoV GI and GII were detected in naturally contaminated oysters and oysters that had been bioaccumulated with sewage. The method permitted detection of 260 genome copies of NoV GII per g oyster gut

(6.6 copies per assay tube), compared with a theoretical limit of detection of 40 genome copies per g oyster gut. The detection limit in genome copies for NoV GI could not be calculated due to the lack of a quantified standard, however the GI assay was highly repeatable and consistent in the detection of the GI standard used, and produced acceptable results in the EURL International Ring Trial/ proficiency test (198). The method allows for 0.025 of oyster gut to be assayed per tube and the equivalent of 0.1g of oyster gut to be assayed for GI and GII for each sample.

### **2.5.2 Cloning and sequencing primer bias**

The primers selected for this study, Mon 431 (195), G2SKF and G2SKR (194) were designed to detect the widest variety of NoV genotypes but had varying homologies for particular genotypes. When used in combination for reverse transcription, cloning and sequencing a mixture of seven GII strains (GII.2, GII.3, GII.4, GII.6, GII.6var, GII.12, and GII.14) a strong bias for GII.4 was observed and, in a mixture of GII.2 and GII.3, a bias for GII.3 was evident. If specific genotypes are to be detected, specific primers for those genotypes could potentially be used. Le Guyader *et al.* (68) proposed that a suite of primers specific for each genotype is needed to enable detection of each genotype. The results of cloning and sequencing NoV from sewage contaminated oysters are discussed in S.4.4.4 and S.5.4.5.

### **2.5.3 HBoV in shellfish and sewage**

HBoV is not as prevalent in faeces as previously predicted (201). Samples from an infectious intestinal disease study (IID 1) conducted in the United Kingdom from 1993-1996 were screened for HBoV using RT-PCR. A small number 7.4% of 2256 samples were found to be positive for the virus, concluding that HBoV is not a significant cause of gastroenteritis (201). This suggests that there is low potential for HBoV to contaminate oyster growing areas. The apparent absence of HBoV in sewage samples tested in this study also suggests that the levels of HBoV in the community considered in Chapter 5 of this Thesis, were low. Because of this low prevalence, whether HBoV will be taken up by oysters was not investigated in this study. If HBoV becomes a public health issue in the future, bioaccumulation studies using oysters and HBoV may be used to resolve this question.



# Chapter 3: A sampling programme for an investigation of potential NoV contamination of oysters in a river estuary

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## 3.1 Preface

This chapter addresses the lack of information worldwide about sampling regimes for the detection of NoV in shellfish and provides an example of a sampling programme for oysters from a river estuary where NoV is at low concentrations and of low occurrence.

## 3.2 Introduction

Two small outbreaks of NoV illness ( $n \approx 14$ ) occurred in people attending functions and were epidemiologically linked with oysters (SRO) from a northern NSW harvest area in 2008 (202). Time delays in notifying the NSWFA of the illnesses meant no faecal samples or leftover oysters were tested. However, the clinical symptoms were consistent with NoV infection and all cases had consumed oysters. The implicated oysters were from a single supplier and harvest area. The harvest area was initially closed by the NSWFA as a precaution. Oysters from the harvest area were sent to New Zealand for testing by Environmental Shellfish Research Institute (ESR) and found positive for NoV GII. Bacterial faecal indicator concentrations were well within the limits for shellfish harvest prior to the outbreaks. Following the identification of NoV GII in the oysters the NSWFA initiated an investigation to identify the potential source(s) of contamination in the area. To assist the NSWFA investigation, a monitoring programme for NoV and *E. coli* in the oysters from that harvest area was developed, undertaken and evaluated as part of this Thesis.

Australian oyster harvest areas are classified according to ASQAP (145). The implicated harvest area, a river estuary, was classified as Conditional Restricted with harvesting only permitted under specific environmental conditions and depuration of oysters was required (203). Information about the possible source of the NoV contamination was limited. Potential sources of raw sewage observed in the harvest area included numerous OSMS, an STP outflow, stormwater drains (SWD) and sewage pump stations (203). NoV testing conducted immediately

after the outbreaks had not indicated a specific source of the pollution, although NoV had been detected throughout the harvest area.

The environmental conditions that may have led to the contamination of the oysters with NoV were unknown. “Routine” conditions for this harvest area were times of medium risk of pollution, such as when rainfall was slightly elevated above normal levels or when there was a higher population in the catchment *i.e.* during summer holidays. “Adverse” conditions for the harvest area were defined as a spill of raw sewage through an STP outfall exceeding 50 m<sup>3</sup> or a large rainfall event *e.g.* > 100mm, during which, significant sewage contamination has occurred. No pre-existing information was available on the presence of NoV in oysters from the harvest area during ‘routine’ or ‘adverse’ environmental conditions.

Exposure of the oysters to NoV was likely to be variable because of several factors including: ‘patchiness’ of pollution due to the river setting; the diversity and disparate location of the potential sources of pollution in the area; and differences in oyster physiology due to environmental variations in the growing area (*e.g.* salinity, turbidity, water temperature etc.). The estuary, a U shaped channel, 4.5m deep, with many bends, has differential flow near the river bank and therefore within oyster racks, which may affect NoV uptake by creating differences in sewage concentrations and residence time around the racks (204). The expected variation in exposure of the oysters to NoV in the area provided the rationale for undertaking intensive sampling of oysters throughout the estuary, including the inclusion of multiple sampling locations (n=7) and collection of five samples from each sampling location. Supporting this decision, large variations in NoV concentrations between naturally contaminated individual oysters and pooled oyster samples have been reported. At very low NoV concentrations, variations in NoV levels were observed between individual Pacific oysters (205) and between pooled samples (16-190 copies GII /g gut) (206). At higher levels of NoV (300-780 NoV GII/g gut), concentrations between pooled samples were more consistent (207).

The aim of this part of the Thesis was to implement an intensive sampling regime to overcome potentially large variations in NoV uptake by SRO, low concentrations of NoV, and to provide information on the spatial and temporal distribution of NoV in SRO in the harvest area during ‘routine’ and ‘adverse’ environmental conditions.



## 3.3 Methods

### 3.3.1 Sampling

Oyster sampling sites were selected after consultation with the Kalang River Premier's Working Group (KRPWG) which was formed under the auspices of the NSW Premier's Department in response to the closure of the Kalang River to recreational fishing and oyster harvesting following the NoV outbreaks. The KRPWG was responsible for coordinating government and oyster industry efforts in facilitating remediation and the re-opening of the oyster production area. The KRPWG consisted of members from NSW Department of Health, NSW oyster industry, NSW Department of Primary Industry (DPI), NSWFA (State shellfish authority), NSW Department of Water and Energy, NSW Department of the Environment and Climate Change, NSW Department of the Premier and Cabinet and local municipal representatives, including waste water engineers and local oyster farmers.

Information about hydrological and environmental influences in the harvest area are documented in Table 3.1. The following factors were considered when deciding the position and number of sampling sites to include in the study: i) locations in which NoV had been detected in oysters previously; ii) areas where high concentrations of faecal indicators (*E. coli*) from known or unknown sources had been evident; iii) the location of commercially active oyster leases; iv) freshwater and tidal influences; v) the quantity of oysters to sustain the sampling regime; vi) the distribution of sites between communities with reticulated sewage systems or OSMS; vii) the funds available to support the testing. Consideration of these factors led to establishment of seven sampling sites throughout the estuary (Figure 3.1). The number of samples of SRO to be collected from each site (n=5) and the frequency of sampling (four sampling events under 'routine' environmental conditions and one sampling event under 'adverse' environmental conditions over seven months) was established to ensure a robust sampling regime to detect potentially low frequencies and concentrations of NoV in the oysters. The sampling plan comprising of five sampling events from seven sites with five samples per site was endorsed by the KRPWG and is described in detail in Appendix A.1.

Samples were collected for each of the five sampling events over one day (n = 35 per day) by local oyster farmers, using a boat and crane to hoist the oyster racks out of the water to enable

collection. All oysters were labelled, bagged and stored on ice before being frozen at -20°C at the end of the collection day. Samples were frozen for logistical reasons because all samples collected in one day could not be tested in a single day.

### **3.3.2 Sampling events**

#### ***3.3.2.1 Illness outbreak sampling 2008***

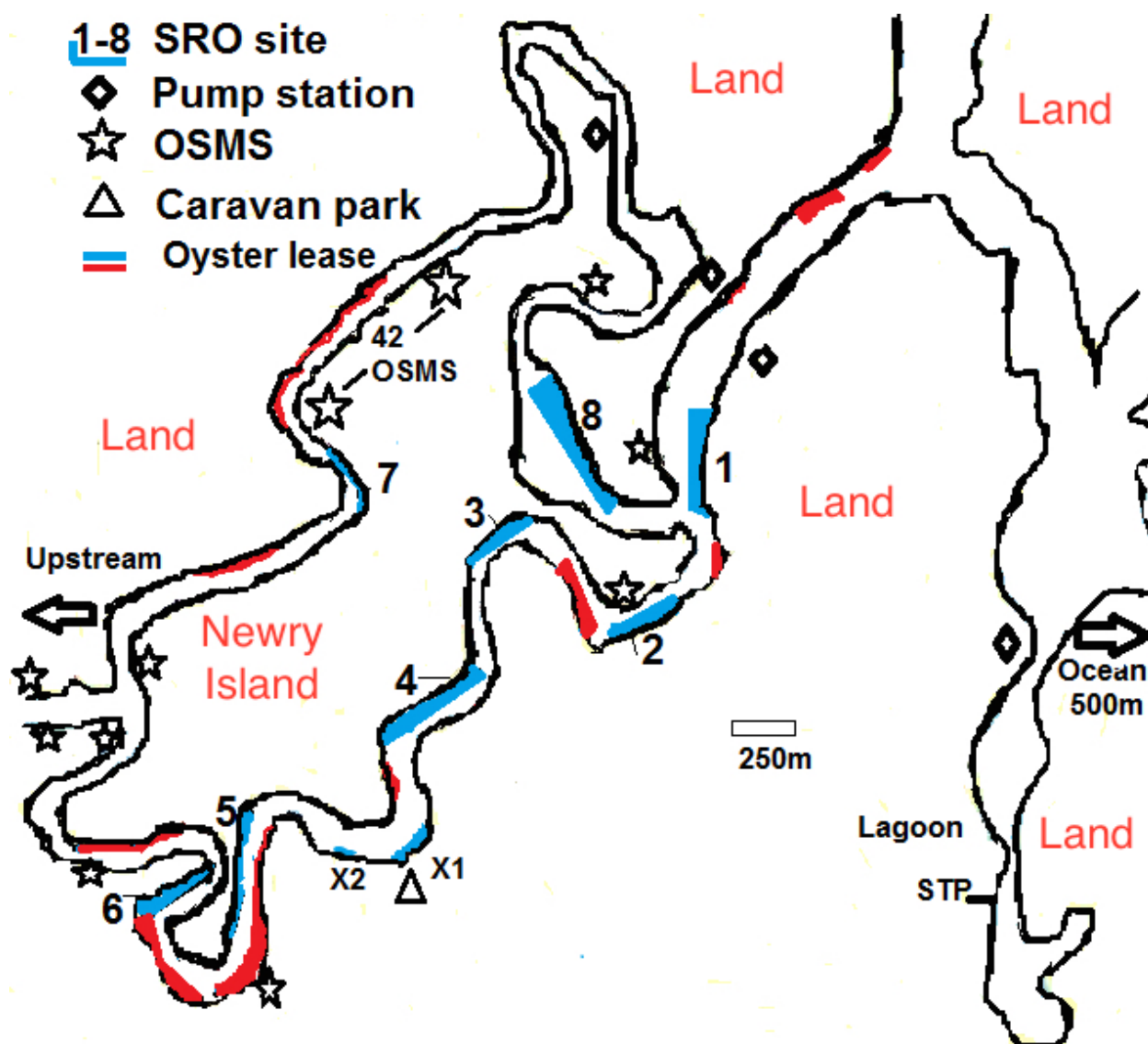
Oysters epidemiologically linked to outbreaks of NoV illness that occurred in July 2008 were sampled by NSWFA from 24<sup>th</sup> July to 28<sup>th</sup> October 2008. These oysters were sent to ESR for testing for NoVGI and GII. Details are provided in Table 3.1 to provide a complete picture of the outbreak testing in 2008 and the study described in this chapter of the subsequent investigation in 2009.

#### ***3.3.2.2 Sampling events 2009***

The ‘adverse’ sampling event (A) was conducted two weeks after a major flood (Flood A, Table 3.1) leading to gross sewage overflows (volume unknown) from the STP and OSMS in the area. ‘Routine’ sampling occasions (R1-4) followed moderate pollution events, R1 (5<sup>th</sup> February 2009) was a week after summer school holidays finished, R2 (26<sup>th</sup> March 2009) was five weeks after flood event A, and 3 weeks after the adverse event sampling and was selected to detect contaminations after the flooding had cleared and the salinity had returned to normal. R3 (5<sup>th</sup> August 2009) followed the mid-year winter school holidays, selected because NoV was more likely to be in the community at this time, and R4 (17<sup>th</sup> September, 2009) was immediately following the spring school holiday period.

**Table 3.1:** River Estuary Environmental Data for 2009

Subject	Details	Reference
Catchment	330 km <sup>2</sup> 150°08'E 36°04'S	(208)
Population	3020	
River use	Oyster farming, recreational – boating, fishing, water skiing, swimming	(209)
Oyster farming	SRO farmed, 36 leases, Harvest area classification: Conditional Restricted, oysters to be depurated prior to sale	
Potential pollution sources	STP: serves approx. 3000, outflow 4 km downstream of harvest area. Several sewage pump stations of concern near waterway. OSMS: in catchment, 122 within 200 m of shoreline, 77 high risk Aerated wastewater treatment systems Marine vessels: small vessels permitted near harvest area Stormwater drains	(203) (154)
Land use	Urban, small areas of farming: dairy; beef; cropping; 36% of land use. State Forest and National Park 63 % land use	(208)
Climate	Subtropical, warm wet summer, mild dry winter	
Solar radiation	Monthly mean for 2009: 9.8 – 23.5 MJm <sup>-2</sup>	(210)
River flow	Mostly derived from groundwater, responsive to rainfall	
Rainfall and Floods	Mostly in summer, average of 1370 mm/ year. Floods (2009): A-159mm in 24h; B-510mm in 24h; C-162mm in 72h	(210)
Tidal Influence	To 23 km upstream	(209)
Tidal prism	Flood tide 0.49 m <sup>3</sup> x 10 <sup>6</sup> main arm 1.54 m <sup>3</sup> x 10 <sup>6</sup> (10km from sea)	(209)
Tidal excursion	Ocean, on a spring tide 9 km, reasonably well flushed	(209)
Max.tidal velocity	10km flood tide north arm 0.28 m/s, main arm 0.48m/s 1-2 m below surface; at bed north arm 0.05m/s main arm 0.15 m/s	(209)



**Figure 3.1:** Diagram of Kalang River estuary and sampling sites

### 3.3.3 Sample handling and transport

Samples were collected and received by the laboratory as planned, with the following exception: five samples per site occurred for samples collected during R1 from site 6, samples 1, 2 and 5 were not collected and during R4, sample 5 was not collected from sites 5, 6 and 7. Samples collected during R2 had thawed on arrival, these oysters were promptly refrozen at  $-20^{\circ}\text{C}$  until testing.

### **3.3.4 NoV testing**

Testing for NoV GI and GII was conducted on oyster gut as described in Section 2.2. The five samples from each site were processed and virus extraction performed together on the same day.

### **3.3.5 *E. coli* testing**

Oysters were frozen prior to sending to our laboratory. R2 had thawed on arrival and oysters were promptly refrozen at -20°C. The method for testing is described in S2.3.7, and allows for testing of frozen shellfish.

### **3.3.6 Additional samples**

Over the period of the study, the growing area was severely impacted by three major floods (details Table 3.1). Some oyster racks and infrastructure (site 2) were washed out to sea following flood A. Site 2 was replaced by site 1. All five sampling positions per site were kept constant throughout the sampling.

Two additional oyster samples, X1 and X2 were taken between sites 4 and 5 for the ‘adverse’ event because a nearby sewage pump station overflowed during Flood A and because of the proximity to a caravan park and three SWD (Figure 3.1).

Oysters were exposed to influent sourced from the STP (Figure 3.1) as described in S.2.3.1.6 to assess whether NoV if present in the influent was taken up by the SRO and also because the method for detection of NoV in sewage had not yet been set up in the laboratory.

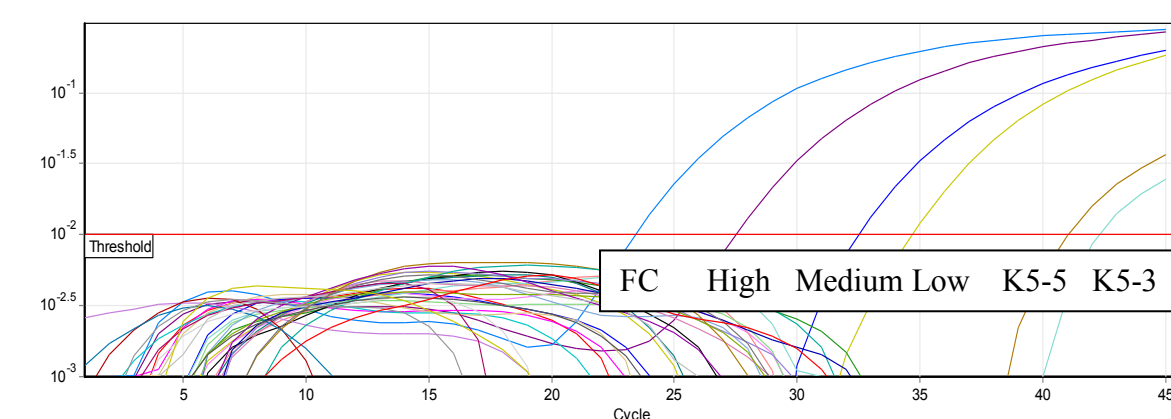
An additional SRO sample was collected as part of R4 from the lagoon near the STP outfall to determine whether NoV was present in the STP effluent in high enough concentrations to be taken up by the SRO.

## **3.4 Results**

### **3.4.1 NoV detection**

NoV GII was not detected in any oyster samples (n=167) collected during the sampling events. NoV GI was detected at all sites tested during R3, (noting that site 7 was not tested for R3). NoV GI was detected in 8.3% of samples (*i.e.* 14 of samples see Tables 3.2 & 3.3). Concentrations of NoV GI detected in the SRO samples collected for R2 in late March and R3 in August, from all sites, were at or above a  $C_T$  of 38.0.  $C_T$  values varied from 38.0 to 44.0 and showed clear

amplification (Table 3.2). Results are detailed in Appendix A:4. Figure 3.3 shows the amplification of samples from R3, site 5 for sample 3,  $C_T$  42 and sample 5,  $C_T$  41. The RLOD had been determined as the lowest concentration of NoV GI at which the RNA standard reliably amplified in 21 out of 24 tubes as described in S.2.4.1.2 and produced an average  $C_T$  of 38.7 with a nominated value of 1 PCRU (raw data are presented in Appendix Figure A: 1). It was possible to amplify lower concentrations of RNA standard and therefore detect lower concentrations of NoV GI, however, the reliability below 1PCRU was not tested. It was not possible to calculate the genome copies for these values, as a quantitative control was not available for GI.



**Figure 3.2:** qPCR amplification profiles (normalised fluorescence vs. cycle number) for NoV GI. Faecal RNA control (FC), RNA standards High, Medium, Low and R3, site 5 samples 5 and 3.

The NoV detections for site 1 samples collected on two occasions are shown in Table 3.3 which also demonstrates the differences evident between samples at the various positions within the site. Site 1 encompassed approximately 300 m of harvest area along the river.

**Table 3.2:** Timetable and NoV detections in SRO samples from 24<sup>th</sup> July 2008 to 17<sup>th</sup> September 2009

Date	24 Jul 2008	14 Aug 2008	28 Oct 2008	5 Feb 2009	16 Feb 2009	2 Mar 2009	26 Mar 2009	31 Mar 2009	21 May 2009	5 Aug 2009	17 Sep 2009
Event	ESR	ESR	ESR	R1	Flood A	A	R2	Flood B	Flood C	R3	R4
Site											
1	NT	NT	NT	NT	Flood	0/5	2/5GI C <sub>T</sub> 38, 38.5	Flood	Flood	1/5GI C <sub>T</sub> 42	0/5
2	NT	1/1 GI & GII	0/1	0/5	Flood	NT	NT	Flood	Flood	NT	NT
3	NT	1/1 GII	0/1	0/5	Flood	0/5	0/5	Flood	Flood	1/5GI C <sub>T</sub> 43	0/5
4	1/1GII	NT	0/1	0/5	Flood	0/5	0/5	Flood	Flood	3/5GI C <sub>T</sub> 41, 41,41	0/5
5	1/1GII	1/1GII	1/1GII	0/5	Flood	0/5	0/5	Flood	Flood	2/5GI C <sub>T</sub> 41, 42	0/4
6	NT	1/1GII	0/1	0/2	Flood	0/5	0/5	Flood	Flood	2/5GI C <sub>T</sub> 44, 43	0/4
7	1/1	1/1GII	0/1	0/5	Flood	0/5	0/5	Flood	Flood	NT	0/4
8	NT	1/1 GII	0/1	0/5	Flood	0/5	0/5	Flood	Flood	3/5GI C <sub>T</sub> 41, 41,44	0/5

NT sites not tested for NoV,   sites <RL0D NoV,   sites and number of positive detections of NoV. R: routine sampling round; A 'adverse' event sampling round, five samples were collected for each site with exceptions, during R1 samples 1,2,5 were not collected from site 6 and during R4 sample 5 was not collected from sites 4,5,6. Results of testing by ESR NZ (24<sup>th</sup> July – 28<sup>th</sup> October 2008) (202) are included for a complete picture of the results following the outbreaks. Flood events (described in Table 3.1) are potentially important for reasons discussed in the text.

#### **3.4.1.1 Controls**

The internal control, aIC was not available for inclusion in the NoV assays for oyster samples from R1. The aIC was included in all other assays as described in S.2.4.1. No inhibition of the aIC qPCR (RNA + aIC) was observed in the samples.

#### **3.4.1.2 Adverse Sampling Event sites X1, X2**

NoV was not detected in oysters sampled from the additional sites X1 and X2 for the Adverse sampling event, thereafter, oysters were not sampled from these sites.

#### **3.4.1.3 Lagoon oyster sample**

NoV was not detected in oyster samples from the lagoon.

#### **3.4.1.4 SRO exposed to sewage**

NoV GII was detected in SRO exposed to STP influent, at a  $C_T$  of 35.9 and equivalent to 1040 copies/ g shellfish gut. NoV GI was not detected in SRO exposed to sewage under the conditions described.

### **3.4.2 *E. coli* results**

*E. coli* concentrations in oyster samples were not related to the presence or absence of NoV in SRO samples (Tables 3.3 & 3.4). *E. coli* concentrations were elevated, > 230 MPN *E. coli*/ 100 g shellfish for the ‘adverse’ sampling event, two weeks after Flood A. Some sites had elevated *E. coli* concentrations after 5 weeks for the R2 sampling event. Table 3.4 highlights the lack of association of *E. coli* with presence of NoV GI in samples from particular positions along site 1. Although NoV GI was detected at site 1, samples from particular positions within the site did not show correlation of NoV GI with *E. coli* concentrations. Variation in *E. coli* concentrations was evident amongst samples from each site during sampling events A and R2 but this was not consistent for each sample on different days, *e.g.* sample 1 did not consistently have more *E. coli* than other samples at site 1, for each sampling event (Table 3.3).



**Table 3.3:** NoV GI detections and *E. coli* MPN for SRO sampled at site 1

Sample and position	1		2		3		4		5	
Test	NoV	<i>E.coli</i>	NoV	<i>E.coli</i>	NoV	<i>E.coli</i>	NoV	<i>E.coli</i>	NoV	<i>E.coli</i>
Event										
Adverse (2/3/09)	≤RLOD	700	≤RLOD	220	≤RLOD	310	≤RLOD	2200	≤RLOD	3500
R2 (26/3/09)	+GI C <sub>T</sub> 38	110	≤RLOD	40	≤RLOD	50	+GI C <sub>T</sub> 38.5	< 20	≤RLOD	70
R3 (5/8/09)	+GI C <sub>T</sub> 42	<20	≤RLOD	40	≤RLOD	40	≤RLOD	<20	≤RLOD	70

  NoV detections, C<sub>T</sub> 38.7 = 1RTPCR/ g shellfish gut, C<sub>T</sub> above this were clear amplifications but could not be quantified., MPN *E. coli* / 100g shellfish 
   <230 MPN *E. coli* per 100g of shellfish, 
   >230 MPN *E. coli* per 100g shellfish

#### 3.4.2.1 Lagoon oyster sample

The MPN was 20 *E. coli* / 100g shellfish for the oyster sample from the lagoon.

**Table 3.4:** Timetable and *E. coli* detected in SRO collected during sampling events in 2009.

Date	5 Feb	16 Feb	2 Mar	26 Mar	31 Mar	21 May	5 Aug	17 Sept
Event	R1	Flood A	Adverse	R2	Flood B	Flood C	R3	R4
Site								
1	NT	Flood	4/5	0/5 GI +	Flood	Flood	0/5 GI+	0/5
2	0/5	Flood	NT	NT	Flood	Flood	NT	NT
3	0/5	Flood	5/5	1/5	Flood	Flood	0/5 GI+	0/5
4	0/5	Flood	5/5	0/5	Flood	Flood	0/5 GI+	0/5
5	0/5	Flood	4/5	1/5	Flood	Flood	0/5 GI+	0/4
6	0/2	Flood	3/5	0/5	Flood	Flood	0/5 GI+	0/4
7	0/5	Flood	0/5	2/5	Flood	Flood	NT	0/4
8	0/5	Flood	4/5	0/5	Flood	Flood	0/5 GI+	0/5

NT :sites not tested,   sites with all samples <230 MPN *E. coli* per 100g of shellfish or ,   number of samples >230 MPN *E. coli* per 100g shellfish, GI+- positive detection of NoV at each site 1-8 in the estuary from 5th February to 17th September 2009. R: routine sampling round; A 'adverse' event sampling round. Five samples were collected from each site with the exceptions of samples 1, 2, 5 from site 6 during R1 and samples 5 from sites 4,5,6 during R4 which were not collected. Flood events (described in Table 3.1) are potentially important for reasons discussed in the text.

### 3.5 Discussion

This study provides an example of a sampling programme for the detection of NoV contaminated oysters when the NoV concentrations and occurrence in oysters are low. Consultation with those familiar with the harvest area, its environs, SRO and the use of historic data enabled selection of sampling sites that were potentially impacted by faecal contamination.

The intensity of the sampling programme, which consisted of five collection events during routine and adverse environmental conditions with five samples collected from each of seven sites over seven months in 2009, enabled the detection of NoV GI in the SRO despite the occurrence and concentration of NoV being low.

As noted earlier, previous studies have shown large variations in NoV concentrations between naturally contaminated individual oysters and pooled oyster samples; at very low NoV concentrations, variations in NoV levels were observed between individual Pacific oysters (205) and between pooled samples (16-190 copies GII /g gut) (206). At higher levels of NoV (300-780 NoV GII/g gut), concentrations between pooled samples were more consistent (207). In this study, at several sites, NoV GI was detected in only one of five samples. Analysis of a single sample instead of five from each site, may have lead to failure to detect NoV. Pooling oyster samples, that have a low concentrations of NoV, may have lead to false negative results (205, 206) because a smaller amount of each individual oyster is tested, meaning that ‘pooling’ can ‘dilute out’ positive samples to a point below the detection limit.

Low concentrations of NoV in shellfish can cause illness. Thebault *et al.* (108) calculated the median ID<sub>50</sub> for GI and GII NoV as 1.6 – 7.51 genome copies per oyster using outbreak data and Le Guyader *et al.* (102) showed illness outbreaks resulting from as little as 100 genome copies of NoV per g of oyster gut. Low concentrations of NoV were detected in oysters from Northern NSW and Dunalley Tasmania which had been linked to illness outbreaks in Australia (97, 106) C<sub>TS</sub> of 38 (NSW) and 41 (TAS) were detected, A. Turnbull SARDI Food Safety, 2015 *pers. comm.*) Thus the detection of low concentrations of NoV in oysters is important from a public health perspective.

The occurrence of NoV in oysters during this study was low, with NoV detected in 14 (8.3%) of the samples (n<sub>total</sub>=167). NoV GI was detected throughout the harvest area, at each of the

seven sites, during a routine sampling event following school holidays. NoV can survive for longer periods in water if it is associated with sediment or particles including plankton (211, 212). However, the detection of GI at each sampled site for R3, albeit at low concentrations, seven and twelve months after GI was reported in SRO from the harvest area by ESR (Table 3.1), suggested a widespread and ongoing contamination. NoV GI strains have been more often linked to shellfish-related outbreaks than GII.4 in the northern hemisphere (68) although Greening *et al.* (100) reported 29 outbreaks of GII and 3 outbreaks of GI linked to shellfish for 2002-2009 in New Zealand. The most recent NoV outbreaks linked to oysters in Australia have been genotypes GII.1 (Dunalley, Tasmania n = 525 cases) and GII.4 (Northern NSW n approx. 32) (97, 106).

The presence or absence of NoV in oyster samples was not related to the concentrations of *E. coli* detected in this study. *E. coli* concentrations were elevated in oysters two weeks after flooding. Some samples had elevated *E. coli* concentrations for up to five weeks after a major flood. It is unlikely that the high concentrations were a result of the flooding of the catchment so long after the flood and may have come from another source. To explain, environmental conditions such as tidal flushing of the harvest area and freshwater input can reduce *E. coli* concentrations in oysters, whereas NoV can be accumulated within an hour of exposure (118) and can remain in the oyster for weeks (119, 137).

This study has illustrated a programme for sampling oysters from a river estuary where NoV is at low concentrations and of low occurrence. The study has shown variation in NoV uptake between SRO samples taken from within the same site and has revealed ongoing sewage contamination of the harvest area.

# Chapter 4 Prevalence of NoV in Australian oysters

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## 4.1 Preface:

This Chapter addresses the lack of experimental data regarding the occurrence of NoV in Australian oysters. The results have been published as Brake F.A., Ross T., Kiermeier A., Holds G., & McLeod C. 2014 “A survey of Australian oysters for the presence of human noroviruses” in *Journal of Food Microbiology* **44** (2014) 264 – 270 (213).

There were several steps in gathering the data. Firstly, the NoV detection method was validated under Australian conditions to ensure detection of low concentrations of NoV in oysters, (*see* Chapter 2). The second involved, the design of a robust sampling regime for the detection of NoV at low frequencies in oysters (*see* Chapter 3). Lastly, the survey described in this Chapter was undertaken encompassing sampling and experimental testing of Australian oysters from a selection of commercial growing areas for the occurrence of NoV.

## 4.2 Introduction

It has been proposed that NoV testing should be incorporated into shellfish risk management programmes partly due to ongoing outbreaks of NoV illness from contaminated shellfish and the inadequacies of *E. coli* as an indicator for NoV contamination (140). The proposed inclusion of NoV testing into shellfish risk management programmes has been driven by the development of standard approaches to NoV sampling and testing. The method, ISO/TS 15216 was recently published and is undergoing interlaboratory validation (134, 135, 214).

In Australia, the conditions for harvesting oysters from all growing areas are regulated by government authorities in each State, following guidance in the ASQAP. Regular monitoring of *E. coli* and/or faecal coliforms in oysters and growing waters is undertaken as part of this program and aims to ensure the microbiological safety of oysters (145). Epidemiological data suggests that the prevalence of NoV in Australian oysters is low (15), however no systematic testing of oysters to determine the prevalence of NoV in Australian oysters has been undertaken.

The aim of this study was to develop a “worst-case” appraisal of the occurrence of NoV and *E. coli* in Pacific oysters or SRO by testing oysters from two of the most microbiologically

compromised oyster-growing areas in each of three Australian States (six growing areas in total) at four different times of the year.

Oyster samples (n=120) were tested for NoV GI and GII using the method of Greening and Hewitt (129) which is closely aligned with the ISO/TS 15216 and has been shown to be both accurate and sensitive for detecting NoV in shellfish, S.2.4 (129, 134, 135) including Australian shellfish (S.2.4). Samples were also tested for *E. coli* using ISO TS ISO/TS16649-3:2005 (E) (196) and Donovan *et al.* (1995) (197)

The data from this survey provides information on NoV and *E. coli* prevalence in Australian oysters at the production level and leads to knowledge that can be used to inform future risk assessment and NoV risk management in Australia.

## **4.3 Method**

### **4.3.1 Shellfish sampling plans**

To develop achievable sampling plans for the selected areas in the three main shellfish producing States, it was necessary to seek assistance from a variety of stakeholders in each State to facilitate sampling activities. Thus sampling plans were developed in consultation with a variety of stakeholders including shellfish authorities in NSW, TAS and SA, members of the oyster industry from each State, SARDI representatives and a shellfish consultant from NZ (B. Hay, AquaBio, NZ). The key considerations of the sampling plans were: (a) statistical rigour; (b) the need to detect very low levels of NoV (and thus multiple samples collected from each area were an integral component); (c) the microbiological quality of the sampling locations; (d) the logistics and practicality of sampling locations; and (e) the finances available (Appendix A.1, A.2, A.3).

### **4.3.2 Shellfish sampling sites and sampling programme**

Six geographically distinct oyster-growing areas (Table 4.1), selected from Australia's three largest oyster producing States NSW, TAS and SA were chosen for this study. All selected sites were considered by shellfish authorities in their respective States as the most compromised with respect to the potential for human faecal contamination as identified from the shoreline surveys and were classified according to the criteria contained in the ASQAP Operations Manual (145). This approach was used to maximize the chances of detecting NoV and *E. coli*, as their occurrence was considered to be unlikely given the lack of human illness outbreaks linked to oysters in Australia.

Table 4.1 gives an overview of each sampling location, including the classification, population in the catchment, seawater temperature, and times of sampling.

For most growing areas, five samples (each sample comprised a minimum of 20 oysters) were taken from each area on four occasions in different seasons over a period of nine months. The NSW 2 sampling site (Table 4.1) however, was sampled over a period of 22 months due to localized flooding coinciding with intended sampling times. Species of oysters sampled from each State reflected the dominant commercially cultivated oysters in each region (Table 4.1).

The five oyster samples collected from each site were taken from geographically diverse sites within the harvest area. TAS 1, TAS 2 and SA 2 growing areas were open for harvesting during each sampling. NSW 1 and NSW 2 sampling sites were closed for harvesting as required by ASQAP growing area classifications (Table 4.1) (145).

Sampling commenced in summer, January 2010 and was completed in early summer, December 2011. Each sample comprised a minimum of 20 oysters. Most of the samples were taken during or immediately following school holidays, when the population had increased up to 3-fold in the respective areas and could potentially place additional pressure on sewage treatment and collection systems. This approach was taken to maximize the likelihood of detecting NoV in oysters.

Outfalls from STPs adjacent to growing areas TAS 1 and NSW 2 were operating within the capacity of each plant. The STP with an outfall adjacent to TAS 1 is a multiple barrier treatment process system that was commissioned in 2005. There had been no noticeable microbiological impact on the TAS 1 growing area from the STP according to faecal coliform results for seawater samples collected by the TSQAP over the previous year (data not shown A. Turnbull, TASQAP, 2013 *pers. comm.*). The STP with an outfall adjacent to NSW 2 was commissioned in 1995 and has an intermittent aeration and decant system with chemical dosing for phosphorous, upgraded with wet effluent polishing through wetlands, followed by UV disinfection. There was, however, an additional 40 year old, secondary trickling filter STP with chemical dosing for phosphorous and upgraded with an effluent pond followed by UV disinfection 4.2 km downstream of NSW 2 which was closed down prior to the final NSW 2, early summer, sampling.

**TABLE 4.1:** Location and parameters of oyster sampling sites for the study

Oyster growing area	Classification as per ASQAP	Species of oysters sampled	Human population within the catchment area	Water temperature °C winter – summer	Flushing rate (tidal cycles)	Sampling dates		Additional information
						Summer	Winter	
						Autumn	Spring	
TAS 1	Conditionally approved	Pacific oysters	2,050	11 – 16 <sup>a</sup>	10 <sup>a</sup>	16/02/10	28/07/10	Situated adjacent to STP outfall
						27/04/10	22/09/10	
TAS 2	Conditionally approved	Pacific oysters	1020	7- 21 <sup>a</sup>	1.4 <sup>a</sup>	16/02/10	29/07/10	
						27/04/10	12/09/10	
SA 1	Closed, inactive	Pacific oysters	200	12 – 24	4.3 <sup>b</sup>	2/02/10	6/08/10	Nursery site for cultivating oyster spat. Water temperature based on local knowledge of the oyster farmer
						4/05/10	2/11/10	
SA 2	Conditionally approved	Pacific oysters	500	12 – 24	4.3 <sup>c</sup>	2/02/10	6/08/10	Water temperature based on local knowledge of the oyster farmer
						4/05/10	11/11/10	
NSW 1	Unclassified	Sydney rock oysters	1,200,000	15 – 25 <sup>d</sup>	1 <sup>d</sup>	27/01/10	18/08/10	Confirmed outbreak of gastroenteritis in 1978-79. The area is used for ongrowing. Oysters are required to be translocated to approved areas for 60 days prior to harvesting.
						20/04/10	27/10/10	
NSW 2	Prohibited	Sydney rock oysters	6500	14 – 27	2-5 <sup>e</sup>	27/01/10	13/07/11	Situated adjacent to STP outfall. The water temperature is based on local knowledge of the oyster farmer. The area is used for ongrowing. Oysters are required to be translocated to approved areas for 60 days prior to harvesting.
						20/04/10	6/12/12	

Data obtained from: a(215), b(216) c(217) , d (218), e (219)



**TABLE 4.2:** MPN of *E. coli* / 100 g of shellfish detected in oysters sampled seasonally from each growing area.

Growing area	Summer			Autumn			Winter			Spring		
	n <sub>230</sub> /n	Median	Max.	n <sub>230</sub> /n	Median	Max.	n <sub>230</sub> /n	Median	Max.	n <sub>230</sub> /n	Median	Max
TAS 1	1/5	90	430	0/5	<20	90	0/5	<20	40	0/5	40	110
TAS 2	1/5	20	310	0/5	<20	40	1/5	70	2400	0/5	<20	<20
SA 1	0/5	20	160	0/5	70	220	0/5	20	130	0/5	<20	<20
SA 2	0/5	20	40	0/5	<20	20	0/5	<20	40	0/5	<20	20
NSW 1	0/5	<20	220	0/5	40	90	0/5 <sup>a</sup>	<20	20	1/5	<20	430
NSW 2	0/5	20	40	0/5	<20	40	0/5 <sup>a</sup>	<20	220	1/5 <sup>b</sup>	70	430

For each season, column 1 indicates the number of samples containing MPN > 230 *E. coli* /100 g shellfish (n<sub>230</sub>) / number of samples (n); column 2 is the median value of MPN *E. coli* /100 g shellfish for each seasonal set of samples; and column 3 is the maximum MPN *E. coli* /100 g shellfish detected in each seasonal set of samples.<sup>a</sup> sample positive for NoV GII, <sup>b</sup> early summer collection

### **4.3.3 Shellfish transportation**

Samples were transported and stored as described S.2.3.1.1 with the following amendments. Most samples arrived at the laboratory within 24h of collection, however, three sets of five samples, TAS 1 (winter), NSW 1 (summer), NSW 2 (summer) were delayed in transit by 24h but were still considered acceptable for analysis because the temperature was only slightly above the desired shipping temperature and the oysters still appeared to be fresh. The temperature of all of the oysters on arrival varied from 4°C to 12°C.

### **4.3.4 Shellfish processing and molecular testing for NoV**

Oysters were prepared, processed and tested for NoV and *E. coli* using the methods described in S.2.3. Oyster extracts that were positive for NoV were sequenced as described in S.2.3.5.

### **4.3.5 Nucleotide sequence accession numbers**

Nucleotide sequences determined in this survey were deposited in the GenBank database under accession numbers KF444835, KF444836 and KF444837.

## **4.4 Results**

### **4.4.1 NoV occurrence**

NoV GI was not detected in any of the 120 samples. NoV GII was detected in two samples (1.7%) from 2 growing areas, NSW 1 site 5 (July 2010) and NSW 2 site 5 (July 2011) that were collected during winter (Table 4.1). NoV GII, detected in the NSW 1 sample, had a  $C_T$  of 31.7 and was quantified as 100,000 copies/g oyster gut. The NSW 2 sample had a  $C_T$  of 41.5, which was quantified as  $\leq 260$  copies/g oyster gut. NoV was not detected in other samples from the spatially distinct NSW 1 and NSW 2 sites collected on the same occasion (*see* Appendix A.3).

### **4.4.2. *E. coli* results**

Five samples (4.2%) collected from growing areas with a variety of flushing rates showed elevated MPN of  $>230$  *E. coli*/100 g shellfish (Table 4.2). None of the samples with elevated MPN *i.e.*  $>230$  *E. coli*/100 g shellfish, had detectable levels of NoV (*see* Appendix A.3).

#### 4.4.3. Control data

Of the 48 qPCR performed for each genogroup for this survey, all provided valid results for the qPCR controls. Ninety percent (43 of 48) of samples had  $R^2$  values  $> 0.98$ . Of the remainder, 2 (4%) had  $R^2$  of 0.97 and 3 (6%) had  $R^2$  of 0.96. One sample from a growing area (SA 2) displayed inhibition of the aIC qPCR (RNA + aIC). The cDNA was diluted 1/10, 1/100 and 1/1000 and retested. A sporadic flow of tannins from decaying native leaf litter into the SA 2 growing area a few days prior to sample collection was observed (K. Lee, PIRSA, 2009 *pers. comms.*).

#### 4.4.4. Sequencing

Three clones were prepared from oyster sample NSW 1.5 and were sequenced. They were all found to be NoV GII.3 and the sequences were deposited in GenBank. These sequences showed variation of only a single nucleotide base substitution within the VP1 gene that was possibly due to PCR error.

### 4.5 Discussion

Oysters tested for NoV contamination in this study were selected from geographically distinct Australian growing areas considered to be the most compromised with respect to the potential for human faecal contamination, as identified from shoreline surveys, in the three main oyster producing Australian States. The low number of NoV detections (two samples) observed in this survey is consistent with the public health record in Australia, which suggests there have been few, and mostly sporadic illness outbreaks linked to contaminated Australian oysters in the past decade (15, 96, 97). One such illness outbreak in 2013, described earlier S.1.4.5 was caused by untreated sewage flowing from a private broken pipe into a Tasmanian oyster growing area (97).

It is of interest that NoV GII was detected in this study whereas NoV GI was detected over a prolonged time in the study described in Chapter Three. Outbreaks of NoV GI are rare in Australia, Bruggink *et al.* (38) reported that during 2002 to 2010 4% of NoV outbreaks were GI and 96% were NoV GII in Victoria. Combinations of GI and GII genotypes accounted for 0.5% of the outbreaks.

The sampling periods in this study were also considered to generally represent times when NoV contamination of waterways would be more likely. Published data on the seasonality of

NoV infections in NSW, TAS and SA is not currently available as NoV is not a notifiable illness in those States. Epidemiological data for the incidence of community-based NoV-associated sporadic gastroenteritis incidents shows a distinct peak of infections during November in Victoria, Australia, however infections of NoV at lower levels continue throughout the year (64). Outbreaks of NoV illness in New Zealand are reported to peak in October (100). Three of our six study areas were sampled (n = 15) during October and November (Spring). Sampling was also undertaken between January and May during or immediately following school holidays to encompass times at which populations in the catchments were higher: up to 3-fold more inhabitants are reported in coastal areas during holiday periods (220).

The occurrence of NoV observed in this study is low in comparison to data collected from similar surveys conducted internationally. Suffredini *et al.* (174) reported that NoV GII.4 and GII.g were detected in 8.3% (n = 120) of Italian shellfish collected from category A and B production areas. Nishida *et al.* (175) detected NoV GI or GII in 9% of Japanese oysters (n = 191) collected from two different production areas. A 2011 study undertaken in the UK (176) notes that 76.2% (n = 844) of samples collected from oyster production areas gave positive results for NoV GI and/or GII. NoV was detected in 63% of all Category A area samples, 78% of category B area samples and in 88% of the samples from Category C. In the USA, 20% of oysters (n = 45) sampled from 33 bays in summer and 12 bays in winter and purchased from farmers, wholesalers and retailers were positive for NoV GII.4. A separate USA survey, however, demonstrated the presence of NoV in 3.9% of oysters (n = 388) collected from retail outlets in nine USA States where 1% (4/388) were GI and 2.8% (11/388) were GII (46, 177). Direct comparisons of the occurrence of NoV in oysters produced in different countries are confounded by the use of different survey and sampling approaches (e.g. retail vs. production area), different shellfish species and analytical methodology. It should be noted that one of the above surveys was conducted using the same analytical methodology to that utilized in this Australian survey: the most recent survey of Lowther *et al.* (176) used ISO/TS 15216 (134) for detecting NoV in shellfish. The reason why the occurrence of NoV in Australian oysters is low when compared to oysters from other countries is not known, however it is possible that the density of the human population residing around oyster growing areas in Australia is lower than in other more densely populated countries.

ASQAP is a guideline that aims to protect the health of the shellfish consumer. ASQAP provides guidance for implementation of sanitary surveys of production areas to identify potential sources of pollution and provisions for the testing of faecal bacteria e.g. *E. coli* and

faecal coliforms, in shellfish and their overlying waters to reduce the risk of human pathogens in shellfish (145). The presence or absence of NoV in oyster samples was not related to the concentrations of *E. coli* detected in this study. Environmental conditions such as the flushing times of the growing areas may have reduced the concentration of *E. coli* in the shellfish whereas NoV, which can be accumulated by the shellfish after an hour of exposure can remain in the shellfish for up to 2 months (118, 119, 137). These results are consistent with similar surveys conducted internationally, which also found that *E. coli* is not always a reliable indicator of the presence or absence of NoV (156, 186). These findings reinforce the suggestion that faecal bacteria may not be appropriate indicators of the presence of human enteric viruses such as NoV in shellfish which has prompted recent calls for the integration of direct NoV monitoring of shellfish into regulatory risk management programmes (140).

A risk-based virus monitoring programme as proposed by EFSA and developed from sanitary surveys of oyster growing areas could improve the ongoing safety of oyster consumers if combined with a focus on preventative measures to avoid contamination of the growing areas with untreated human faeces (89). However, the low number of NoV detections obtained in this oyster survey underscores the importance of monitoring programmes being introduced into quality programmes on a risk basis.

The results of this survey will contribute to risk management strategies for Australian shellfish authorities and be useful for future risk-based assessments of the potential for NoV contamination of Australian oyster growing areas.



# Chapter 5: Spatial and temporal distribution of NoV and *E. coli* in SRO following a sewage overflow into an estuary

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## 5.1 Preface:

Preceding chapters have described the validation of a detection method for NoV in Australian oysters, a programme with which to sample oysters when NoV is in low concentrations and of low occurrence and a survey estimating the prevalence of NoV in Australian oysters. This chapter addresses the lack of information about the spatial distribution of NoV in oysters after sewage overflows and the persistence of NoV in SRO by mapping the concentration of NoV in SRO after a sewage overflow in an estuary.

## 5.2 Introduction

In the event of a sewage overflow, shellfish authorities use a variety of strategies to minimise the human health risk from bivalve consumption, including the closure of harvesting areas to allow sufficient time for inactivation and elimination of human pathogens (169, 221). Two major considerations when closing harvesting areas following sewage spills are: (a) the delineation of the area that has been affected by the spill; and (b) the amount of time that human pathogens can persist in a viable state within bivalves. To evaluate these considerations, regulatory authorities have traditionally tested shellfish and/or the growing waters for faecal bacterial indicators such as *E. coli*. However, faecal bacteria are reported (149, 151, 152) to depurate from shellfish within days, whereas, NoV has been detected in shellfish intermittently for up to eight weeks (119). Additionally, faecal bacteria are more susceptible to environmental stressors such as solar radiation than viruses (149), which may limit the distance that viable indicator bacteria can be dispersed within water bodies when compared to NoV (159). Given the major differences in the behaviour of faecal bacteria and NoV within water bodies and shellfish, an understanding of the distances over which viruses can be dispersed and the time they persist in a viable state within bivalves is critical for calculating appropriate closure areas and the duration of closure.

The distances over which NoV GII can be dispersed has been studied previously using PCR-based methods of detection. NoV GII was detected in mussels that were located 10 km away from a large primary sewage treatment plant (STP) outflow in New Zealand (166). Similarly, in Mobile Bay, Alabama, USA NoV GII was detected in Eastern oysters that were located 5.74 km away from the discharge point of a wastewater treatment plant. Dilution and dispersion of rhodamine dye-tagged sewage was used and effectively determined the path of the effluent (159). Ueki *et al.* (157) detected NoV in oysters approximately 1 km away from an STP outflow. Oysters in a harvest area 570m from a combined sewer overflow (CSO) showed a slight increase in NoV GII from < RLOD 3h prior to the discharge to 3150 copies/ 100 g shellfish within 12 h, levels that were maintained for 74h (160). Greening *et al.* (119) studied the persistence of NoV in Pacific oysters that were contaminated with the virus via an in-tank bioaccumulation process and then placed in the marine environment, and NoV RNA was detected by real-time PCR for up to 4 weeks and intermittently for 8 weeks by semi-nested PCR.

Apart from these few studies, there is very little empirical field data regarding the time that NoV persists and the distance over which the virus spreads, particularly for different types of shellfish species, water bodies and environmental conditions. Data describing the temporal and spatial distribution of NoV in SRO after a sewage contamination does not exist. The lack of data may result in closures unrelated to the human health risk to oyster consumers because the period of closure does not accurately reflect the time that viruses are retained in SRO or spatial distribution of viruses in SRO harvest areas.

Given the foregoing, the main aim of the study described in this Chapter, was to provide data about the spatial and temporal distribution of NoV, HAV and *E. coli* in SRO following a pump station sewage overflow in an estuary in northern NSW used for farming oysters. These results of this study will support shellfish authorities to derive appropriate closure areas and times and assist in minimising the public health impact of sewage contamination events.

### 5.3 Methods

An estuary in northern NSW was selected for the study because:

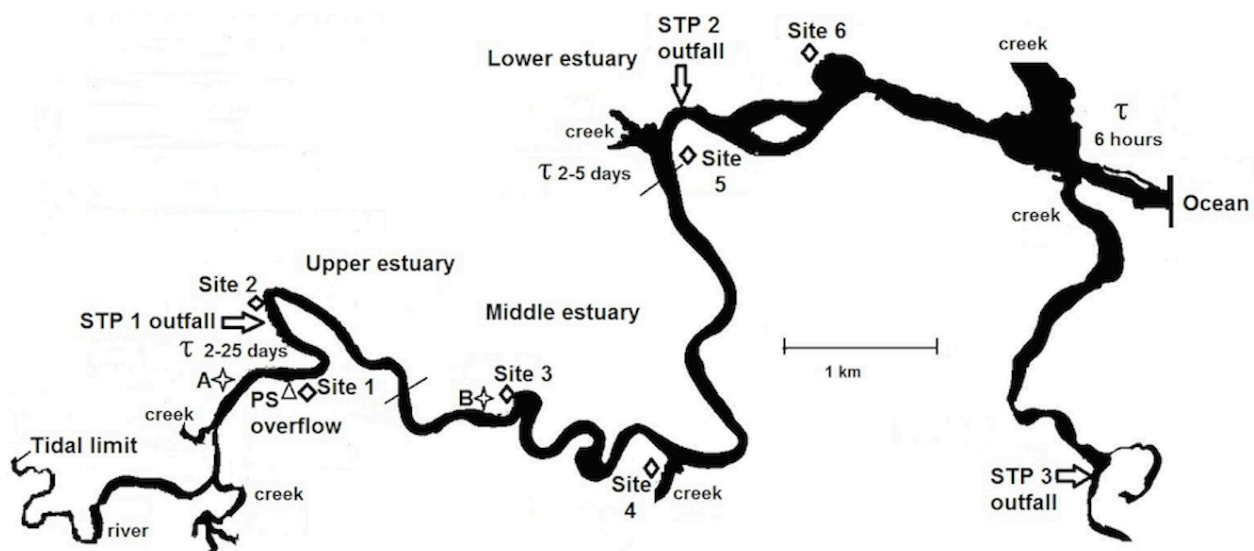
- i) oysters were commercially grown within the estuary;
- ii) a pump station located at the upper edge of the estuary, 600m upstream of the STP, was known to overflow during high rainfall events



- i) hydrodynamic and environmental impact studies of the estuary were available (219, 222-225).

Oysters were installed at five sites in the estuary prior to the wet season (Table 5.1 and Figure 5.1). The sites spanned the distance (8.2 km) between the pump station and a commercial oyster farm that was located close to the sea. Following the installation of the oysters, 75 days elapsed until a significant overflow of sewage occurred from the pump station. Oyster samples were then collected from each of the five sites and the oyster farm for up to 48 days to study spatial and temporal distribution of NoV, HAV and *E. coli*.

Characteristics of the estuary are listed in Table 5.1. One third of the catchment is forested, the rest of the estuary is vegetated nature reserves, cleared rural land and three towns near to site 1 and either side of the river at site 6 (Figure 5.1). Three STP serviced the towns, STP 1 and 3 were decommissioned shortly after the completion of the study (Figure 5.1).



**Figure 5.1:** Map of estuary. Δ Pump station (PS) overflow, ◇ Oyster sampling sites, ⇨ STP outfall, τ Flushing times, ☆ Council testing site, \ Estuarine reach

STP 1 was located on the main arm of the river and was a conventional trickling filter plant with chemical dosing with phosphorous to provide secondary sewage treatment. The plant was commissioned in 1963 and was designed to service 3000 people in predominantly dry weather with an average of 1 ML per day going through the plant. STP1 was upgraded in 2000 to include an effluent pond and UV disinfection prior to release. Sewer pipes were separate to stormwater drains. The pump station associated with the overflow was one of 12 pump stations connected to STP1 and was the only station to overflow (Figure 5.1) during the study. The estimated maximum flow from the pump station to STP1 was 41.8 L/s (224). The pump station was upgraded after the completion of this study.

A tertiary treatment plant, STP 2 with intermittent aeration and decant, chemical dosing for phosphorous and wet effluent polishing through wetlands, followed by UV disinfection, was installed within the estuary catchment in 1995 (Figure 5.1). STP 3, a 40 year old plant located down a small tributary stream was the same type as STP 1 with similar upgrades. The overflows documented in Table 5.1 for STP1 are the only overflows or bypasses to have occurred during the study period (226-228).

**Table 5.1: Estuary characteristics**

Characteristics	Details	Reference
Estuary area	2.9 km <sup>2</sup> , entrance permanently open	(223)
River length	20 km	
Estuary catchment	228 km <sup>2</sup> , one third of area is forested	(222)
Volume	4267 ML	(222)
Tidal prism	1200-1400 ML	(222)
Tidal input	14 km up main arm	(223)
	Influenced by spring and neap tides (0.44 m site A Figure 11)	
	Flooding and storm surges more important for water exchange in upper estuary, up to 14x flow	
Flushing time	Upper estuary: up to 20 days and < 1 day in high flows	(219)
	Middle and lower estuary: 2-5 days	
Latitude and Climate	28.56°S, subtropical, large episodic downpours with extended periods of no or low rainfall	(225)
Daily solar exposure	Over the study period, 2.8 MJ/m <sup>2</sup> to 23.3 MJ/m <sup>2</sup>	(229)
	Mean of 15.4 MJ/m <sup>2</sup>	
Population	Upper estuary 3500, total catchment 11,000	(220)
	Population increases 2 fold in summer (Dec-Feb) & holiday periods.	
Estuary use	Recreational: fishing; boating; swimming. commercial: fishing; farming: oysters; cattle.	

### 5.3.1 Oyster sampling sites

Five oyster-sampling sites were installed for the purposes of this study (Figure 5.1, sites 1- 5). An oyster farm located 2.4 km from the ocean served as a sixth sample site. Site 1 was installed

as near as practical (50 m downstream) to the anticipated pollution source, *i.e.* the overflow pipe. Site 2 was located approximately 650 m from the pump station overflow and 50 m downstream of the STP1 outflow. Site 3 was located 1.93 km from the pump station overflow. Oysters located at site 4, 5.29 km from the pump station were positioned slightly off the main arm of the river and 30 m into a creek where oyster leases had previously existed. Site 5 was an unused oyster lease located 6.82 km from the pump station. Site 6, a commercial oyster farm, was located 8.20 km downstream of the pump station, 1.38 km downstream of the STP2 outflow and 4.2 km from the STP3 outflow (Figure 5.1).

SRO cultivated at site 6 were used to stock sites 1-5. Oysters from the farm at site 6 are not sold directly to the public, they are required to be translocated to clean waters for 60 days prior to sale for human consumption (145). Oysters were harvested from site 6 and installed at sites 1-5 on 5<sup>th</sup> May 2010 *i.e.* 75 days prior to the pump station overflow. A sample of these oysters was tested for the presence of NoV GI and GII.

Oysters placed at sites 1- 6 were contained in either plastic net tubes (50 oysters per tube), or bags (25 oysters per bag), and attached onto poles fixed in the river two metres from the water's edge. The net containers were set at a depth appropriate for inter-tidal cultivation. A total of 160 oysters were installed at each of sites 1 – 6. The six sample sites extended 8.2 km downstream from the pump station associated with STP1, which was located 10.6 km upstream from the ocean.

### **5.3.2 Sample collection and transportation to the laboratory**

Oysters were collected from each sample site by boat at approximately weekly intervals (days 5, 12, 20, 28, 34, 42 and 48) between 2<sup>nd</sup> August 2010 and 14<sup>th</sup> September 2010 following the sewage overflow event on 28<sup>th</sup> July 2010. Twenty oysters were sampled from each site on each sampling occasion. Samples were dispatched to the laboratory as described in S.2.3.2.1. Samples from day 5 and 28 were delayed in transit by 24 hours but had been kept cool (4°C) during the delay. HAV analysis was conducted at a later date using extracted RNA, which had been stored at -80°C. Sewage grab samples obtained from STP1 consisting of one litre of STP influent and effluent were collected on day 7, after the influx of rain. The sewage samples were transported to the laboratory on ice and immediately frozen to -20°C on arrival, prior to analysis.

### 5.3.3 Analysis of oysters and sewage for NoV and HAV

The method for extraction and detection of NoV and HAV in oysters and sewage was described in S.2.3.1.2-3 and S.2.3.4.

### 5.3.4 Sequencing

NoV detected in oyster samples from day 1- site 1, day 12 - site 1 and day 12 - site 6 and sewage influent were further characterized by cloning and sequencing as described in S.2.3.5. Clones were prepared from the oyster samples which were collected on day 5-site 1 (7 clones) and day 12-site 1 (1 clone) and were sequenced. Nucleotide sequences determined in this study were deposited in the GenBank database under accession number KM 260221.

### 5.3.5 Enumeration of *E. coli* in oysters

For each oyster sample, homogenates were tested for *E. coli* as described in S.2.3.7.

### 5.3.6 Statistical analysis of results

The relationship between (i) the concentration of NoV or *E. coli* in the oysters and distance of the oysters from the pollution source; and (ii) the elimination of NoV or *E. coli* from the oysters with time were evaluated statistically. NoV and *E. coli* results were  $\log_{10}$  transformed for the analysis, which was undertaken using R, version 3.0.2 (R Core Team, 2013). Significance was assessed using a significance level of 0.05 and non-significant effects were removed, one at a time and observing marginality. The model assumptions were assessed using standardized residual plots and normal quantile plots. Because of the  $\log_{10}$  transformation, estimates of the slope are interpreted as percentage increases/decreases in NoV or *E. coli* concentration.

A response surface model was fitted for distance and time after the spill, nested within distance, to  $\log_{10}$  *E. coli* (MPN/g) data. Seven results above the upper limit of detection (uLOD) were treated as being equal to  $\text{uLOD} = 18,000$  MPN/g and similarly one result below the lower limit of detection (ILOD) was treated as equal to  $\text{LOD} = 20$  MPN/g.

To assess the effect of distance from the pollution source on the concentration of NoV in the oysters, a separate regression model was fitted to the  $\log_{10}$ NoV concentration data using the day 5 data for sites 1 to 4 (only) with distance (m) as the explanatory variable. Sites 5 and 6 data were not included in the calculations because NoV was not detected at these time points.

Secondly, an evaluation was undertaken to determine if NoV or *E.coli* elimination from oysters over time following the sewage overflow was significant. For log<sub>10</sub>NoV only site 1 and 2 had enough NoV detections (over time) to allow fitting a model with time as the explanatory variable. A separate regression was fitted to the data from each of the two sites.

For comparative purposes, previously published changes in NoV concentrations in oysters over time from:

- Figure 2 in Dore *et al.* (137)
- Figure 1 from Greening *et al.* (119)
- Figure 7, from day 2 to day 14, from Neish (138) and also

HAV concentrations in oysters from Kingsley and Richards (230) were estimated from graphs using WebPlotDigitizer (<http://arohatgi.info/WebPlotDigitizer/>). A linear regression model was fitted to the estimated log<sub>10</sub> transformed NoV or HAV concentrations to assess the reduction in NoV or HAV over time.

## 5.4 Results

### 5.4.1 Sewage overflow and hydrology

Shellfish management authorities and SARDI laboratories were notified of a metered sewage overflow of 3021.9 kL over 21.25 h (142.2 kL/h) from the pump station (Figure 5.1). Details are presented in Table 5.2 as a calendar of events. The sewage overflow, which commenced on 28<sup>th</sup> July 2010, was taken as “day 0” for laboratory purposes.

The dilution of the sewage overflow into the entire estuary, not including tidal or freshwater inputs, was 1: 1415. The dilution of the overflow at site 1 for the duration of the overflow was calculated as 1: 5.2 using the volume of the sewage overflow / volume of freshwater input. The dilution of the overflow at site 2 included the volume of STP effluent (Vol of overflow/ freshwater input + STP effluent) and was 1: 7.9. These are ‘worst case’ (*i.e.*, lowest dilution) estimates for the dilution of the sewage overflow event because they do not include the influence of tide nor the volume of receiving waters in the estuary. These calculations are used by the local council in response to requirements of the NSW EPA Environment Protection License for STP1 (231, 232) (D. Baulch, Acting Manager Utilities, Infrastructure Services, Byron Shire Council

2014 *pers. comm.*). Spring and neap tides also influenced the estuary because day 0 was two days after a spring tide (Table 5.1).

**Table 5.2:** Calendar of events: rainfall; freshwater inputs; and sewage overflows in estuary

Day	Metered sewage overflow (kL)	Rainfall (mm)	Fresh- water input (ML)#	Flushing time (upper estuary) (days)	Details	Ref.
75 days prior to day 0					Oysters installed‡	
55 days prior to day 0	4082*	83	154	1-2		(222, 225, 233, 234)
1 day prior to day 0			9			(233)
Day 0	3021.9	105	25	4-7		(222, 225, 233, 234)
Day 6			14		Oysters sampled	(233)
Day 12			10		Oysters sampled	(233)
Day 13	292.7	10	25	2-3		(222, 225, 233, 234)
Day 14		52	70			(233, 234)
Day 20			NA		Oysters sampled	(233)
Day 24		7	17			(233, 234)
Day 27		6	14			ib sit
Day 28			14		Oysters sampled	(233)
Day 34			10		Oysters sampled	ib sit
Day 39		2	8			(233, 234)
Day 42			8		Oysters sampled	(233)
Day 44		8	8			(233, 234)
Day 48			8		Oysters sampled	(233)

# Input at gauging station 9.5km upstream of site 1, representing 71% of freshwater into upper estuary,‡ oysters  $\leq$  RLOD for GI & GII.\* no oysters sampled as pollution events prior to installation may have influenced NoV detection, NA- data not available

**Table 5.3:** Salinity and water temperature for Council sampling sites A, B & 6

	Site A	Site B	Site 6	References
Annual salinity (psu)	0.4 – 0.6	5 – 10	16 – 29	All data from D. Baulch Acting Manager, Utilities, Infrastructure Services, Byron Shire Council, NSW 2014
Water T °C on day 0	16	16.3		
Water T °C	18.8– 21.8	19.1– 22.4		
Sep 12-17 2003-2010	Av. 19.7	Av. 19.6		

Water collected from site 1 (Figure 5.1) on installation of the oysters, had a salinity of <1psu assessed using a handheld conductivity meter (WTW, Germany). SRO's are halotolerant organisms; and can tolerate a wide range of salinities and temperatures (235). The oysters sampled from site 1 were healthy (Figure 5.2). Salinity and water temperature data was recorded by local municipal officers however the water temperature was not recorded after day 0. As a surrogate, data collected over the previous 9 years between 12<sup>th</sup> - 17<sup>th</sup> September (last week of study) was averaged (Table 5.3).

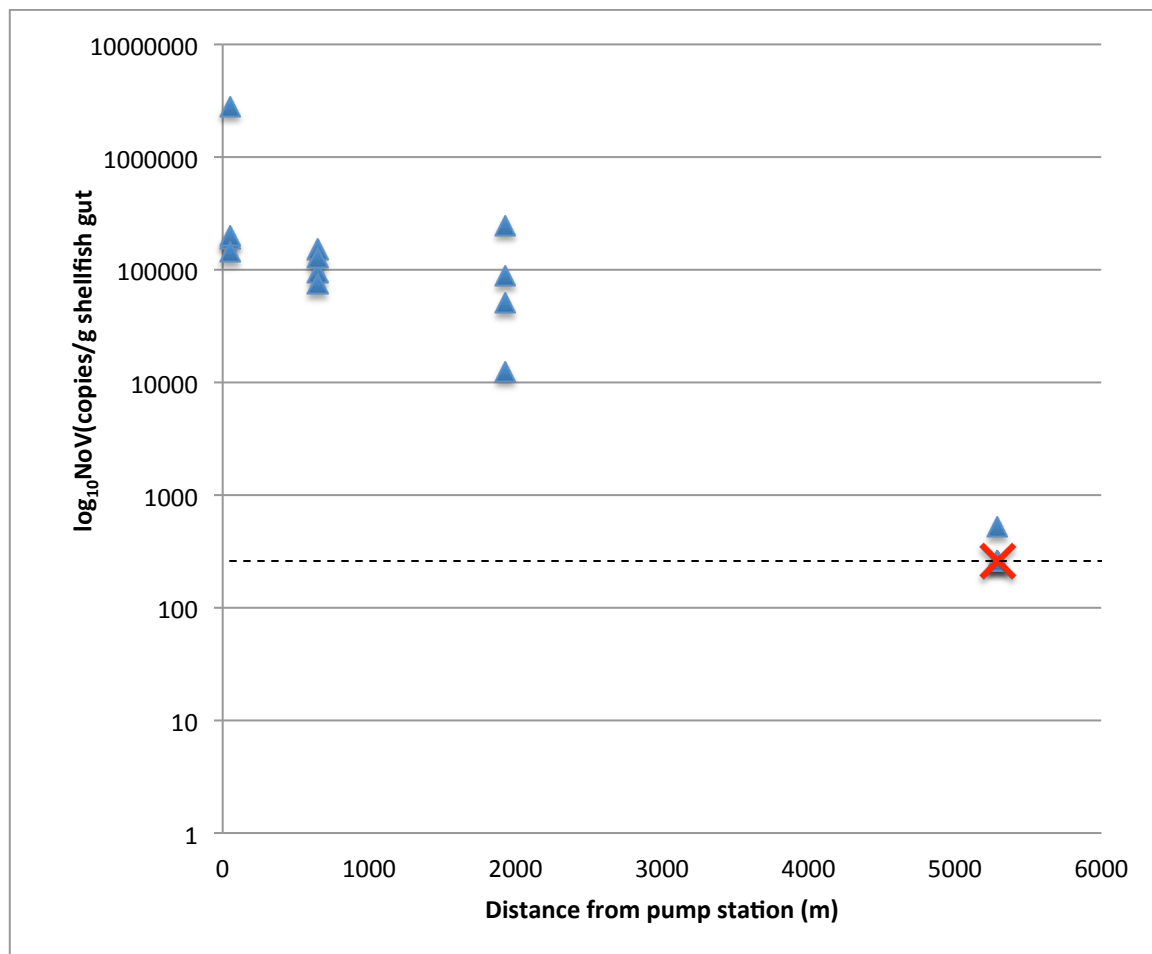


**Figure 5.2:** Photographs of shucked SRO (Left) at installation, (Right) day 5 from site 1. SRO were healthy after 80 days at site 1.

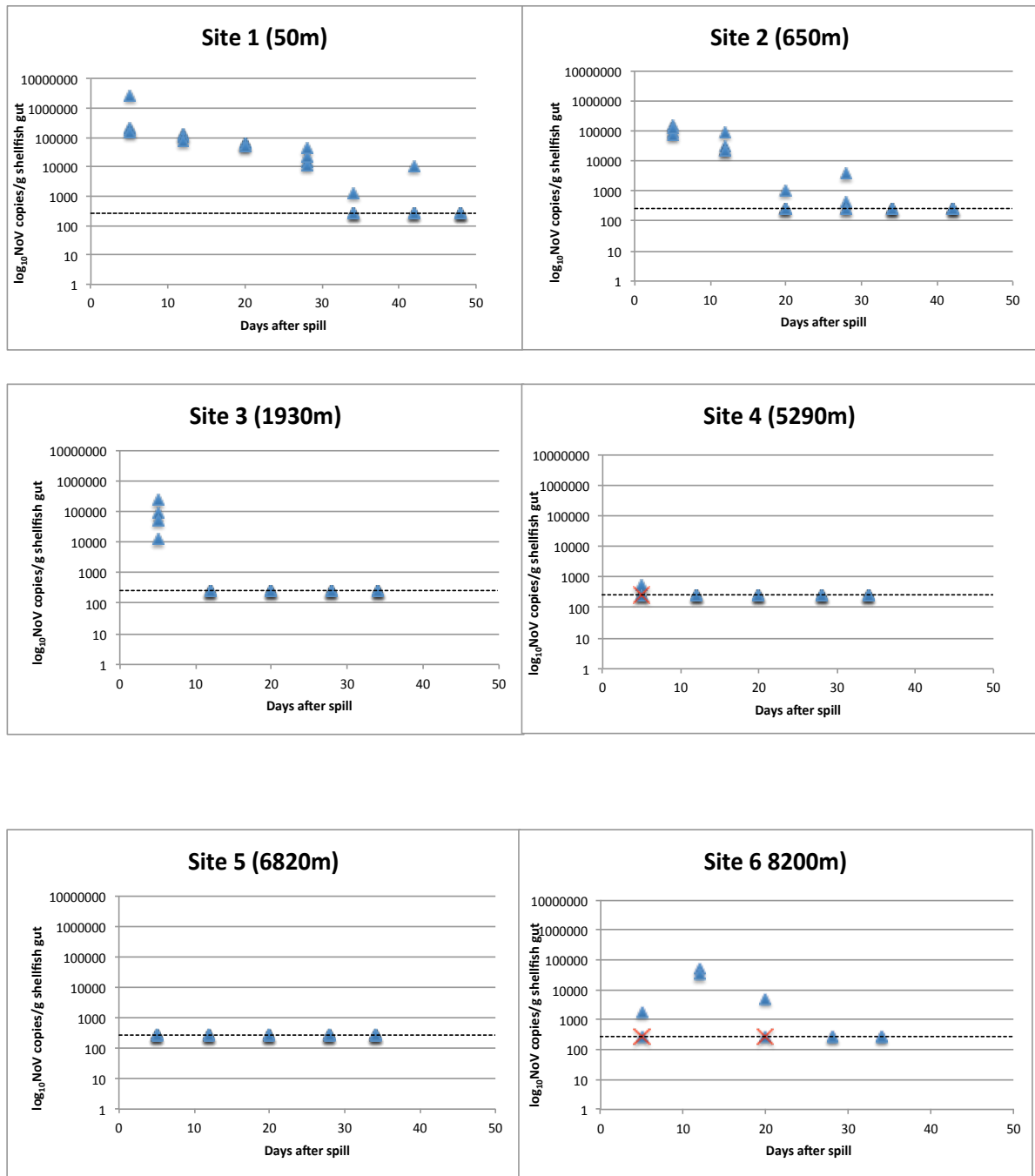


### 5.4.2 NoV and HAV occurrence

NoV GII was detected in oysters on the first sampling occasion, day 5 at sites 1- 4, which were between 0.05 and 5.29 km from the pump station overflow (Figure 5.3), but it was not detected at site 5. The concentrations of NoV GII in oysters on day 5 at sites 1- 4 decreased with distance ( $p<0.001$ ) at a rate of 5.8% per kilometre from the sewage overflow. At site 6, 1.38 km downstream of the STP 2 outfall, NoV GII was detected on days 5, 12 and 20 (Figure 5.4).



**Figure 5.3:** Scatter plot of individual qPCR test results as  $\log_{10}$  NoV GII copies/g shellfish gut versus distance (m) from the sewage overflow on day 5 for sites 1 - 4. The dashed line represents the RLOD (260 NoV GII copies/ g shellfish gut). Test results noted in blue and situated on the dashed line were  $\leq$ RLOD (not detected). **X** were detects  $\leq$  RLOD, marked on the dashed line.



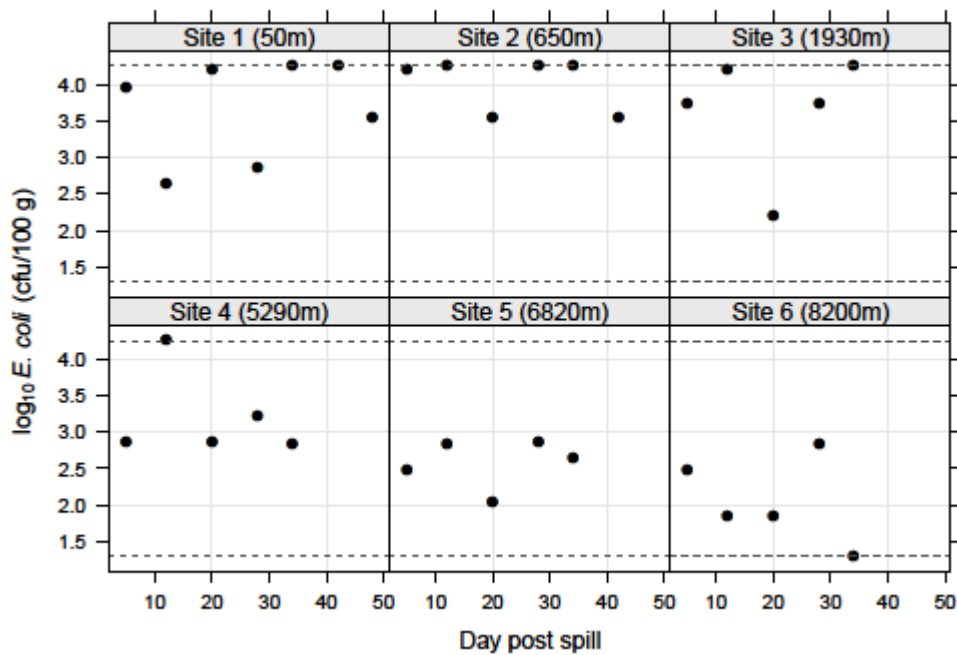
**Figure 5.4:** Scatter plots of individual qPCR test results as  $\log_{10}$  NoV GII copies/g shellfish gut versus time (days) after the sewage overflow for each sampling site 1-6. Four individual qPCR test results were recorded for each sample collected from sites 1-5 and two for site 6 due to the number of spaces in the Rotorgene Q. The finely dashed line represents the RLOD (260 NoV GII copies/ g shellfish gut). Test results noted in blue and situated on the dashed line were  $\leq$ RLOD (not detected). **X** were detects  $\leq$  RLOD, marked on the dashed line.

NoV GII was detected in oyster samples for 42 days after the overflow at site 1 and for 28, 12 and 5 days after the overflow at sites 2, 3 and 4, respectively (Figure 5.3). For sites 1 and 2, the NoV concentrations in oysters decreased over time at a rate of 8.5% per day ( $p < 0.001$ ) (Figure 5.4). At sites 3 and 4, NoV was detected on day 5 but NoV concentrations were reduced to  $\leq$ RLOD by day 12. Individual qPCR results are provided in Appendix A:6. There was no noticeable effect of a second, smaller sewage overflow from the same pump station, that occurred 13 days after the initial large overflow event (*e.g.* concentrations of virus did not further increase). Virus if present, may have been washed quickly downstream with a larger input of freshwater flowing down through the estuary and out to sea (Table 5.2).

NoV GI was not detected in any of the oyster samples collected from each site on day 5 and 12 and hence no further testing for GI in oysters was conducted after day 12. While NoV GI was not detected in the oysters, low levels of NoV GI were detected in the STP influent (260 PCR Units/ L:  $C_T$  38.4) and effluent ( $C_T$  40,  $\leq$ RLOD). NoV GII was detected in the STP influent at 46,000 genome copies/ L ( $C_T$  36.2) but was not detected in the effluent. The presence of HAV was tested for, in oyster samples collected on day 5, 12 and 20 from sites 1 and 2, day 12 site 6 and sewage influent was assessed but was not detected.

#### **5.4.3 *E. coli* results**

The MPN of *E. coli* /100g of oysters sampled on day 5 from sites 1 - 4 were found to decrease ( $p < 0.001$ ) with distance from the pump station sewage overflow at a rate of 20.1% per km. *E. coli* concentrations did not decrease significantly over time ( $p = 0.567$ ). Of note, oysters with elevated MPN of 18,000 *E. coli* / 100 g shellfish were detected sporadically as far as site 4, 5.29 km downstream of the pump station overflow and after 42 days at site 1 (Figure 5.5). At site 6, *E. coli* concentrations fluctuated up to 700 *E. coli* / 100 g shellfish (Figure 5.5).



**Figure 5.5:** Scatter plots of MPN  $\log_{10} E. coli$  /100g shellfish versus time (days) after the sewage overflow for each sampling site. The upper dashed line indicates the upper limit of detection (18,000 MPN/100 g shellfish). The lower dashed line indicates the lower limit of detection (20 MPN/100g shellfish).

#### 5.4.5 Sequencing

RNA sequences derived from oysters collected from site 1 on day 5 and 12 were found to be NoV GII.12. For two of the clones the sequences were found to vary by one or two bases only, in sequences of 302 bases, possibly due to PCR error. The sequence of the dominant clone was deposited in GenBank. NoV from oysters collected from site 6 or from the sewage influent or effluent extracts which were positive for GI and/ or GII using qPCR were not able to be sequenced.

## 5.5 Discussion

This is the first study to describe in detail NoV GII distribution and persistence in SRO located in an estuary after a pump station sewage overflow. Under low salinity conditions, NoV GII was taken up by the oysters following the overflow. The virus was detected in oysters situated 5.29 km downstream of the outfall and persisted in oysters for 42 days at the most ‘impacted’ site -1, *i.e.* 50m from the pump station and 550m upstream of the STP outfall. While NoV was also detected in oysters at site 6, it may have originated from either the STP 2 outfall, slightly upstream or the STP 3 outfall as no NoV was detected at site 5. STP 1 and STP 3 were both decommissioned shortly after this study and the source of the sewage overflow, the pump station, was refurbished.

Concentrations of *E. coli* and NoV in the oysters decreased significantly with increasing distance from the pump station outfall. This is likely to be related to higher dilutions of these microorganisms due to further freshwater inputs downstream, related to run-off in the lower catchment, and larger tidal influences at sites closer to the sea. The relative decrease in *E. coli* concentrations in oysters with increasing distance was larger than that observed for NoV GII (20.1% per km vs. 5.8% per km). This finding likely relates to the higher stability of NoV compared with *E. coli*. *E. coli* can be eliminated from oysters and inactivated in water within a few days due to tidal and environmental influences (149-152) whereas NoV is more robust in the environment (50) and can persist in oysters for weeks (119, 137). The lack of correlation observed in this study between the presence of NoV and *E. coli* reinforces previous observations (156, 176, 236).

A NoV GII reduction rate from the oysters of 8.5% per day was observed. Greening *et al.* (119) investigated the reduction of NoV in Pacific oysters, following in-tank bioaccumulation of NoV GII.3 and subsequent placement of the oysters in the sea. Based on data in Greening *et al.* (119), the estimated NoV reduction rate was 9.5% per day. Dore *et al.* (137) tested naturally contaminated Pacific oysters for NoV GII that were relayed into a clean growing area, from which there was an estimated NoV reduction of 4.5% per day during the relay period. Neish, (138) followed the reduction of NoV GII from Pacific oysters in depuration tanks at 16°C and 8°C. The oysters had been bioaccumulated with the virus five days prior to commencing the study. The estimated NoV reduction rate from day 2 was negligible from oysters in depuration tanks at 8°C and 3.9% from oysters in tanks set at 16°C.

The water temperatures during these four studies showing reduction of NoV from oysters, were from 16°C rising to 19.6°C for the current study, from 18°C falling to 12°C (mean of 14°C) (119), between approx. 15 – 17°C (W. Dore, 2014 *pers. comm.*) and 16°C (138) respectively. The reduction rates from this study, Greening *et al.* (119), Dore *et al.* (137) and Neish (138) are similar despite different detection methods, environmental conditions, oyster species and NoV GII genotypes (GII.12, GII.3, unknown and unknown respectively). The data presented here is based on qPCR which does not necessarily indicate the viability of NoV, however it is interesting that a reduction rate of 8.95% per day was also estimated for viable HAV from Kingsley and Richards, in which HAV was shown to persist for three weeks in Eastern oysters using a cell culture method (230). The similar reduction rate estimates for NoV GII may be useful to inform future calculations on the time needed for contaminated oysters to eliminate the virus to levels that are negligible in terms of public health risk.

These results demonstrate significant levels of accumulation of NoV GII by oysters within five days of a sewage overflow, despite very low salinity conditions. This finding is consistent with a previous study that shows accumulation of NoV in 90% of shellfish tested two days after a hurricane (237) where raw sewage and large volumes of fresh water had affected the shellfish. Walne (238) showed that filtration rates of the Eastern oyster *Crassostrea virginica* returned to normal within six hours when the salinity was suddenly reduced from 27 psu to 5 psu, and that Pacific oysters increased filtration rates when the flow rate of the growing waters increased, presumably to maximize nutrient intake.

The dilution factor of the overflow into the entire estuary was estimated to be 1:1415. The dilution factor at each site is unknown and could not be determined from available data. However the upper estuary sites were exposed to higher concentrations of sewage than the lower estuary sites. Our findings are consistent with those of Goblick *et al.* (159) who demonstrated that NoV from STP effluent was detected in Eastern oysters located 5.74 km away from the STP outfall and showed that the dilution of effluent in water ranged from 1:158 to 1: 556. Collectively, these results confirm that NoV can be dispersed over large areas within estuaries and could potentially expose oyster consumers to risk of viral infection. Internationally, there has been discussion about the use of sewage dilution factors to guide the closure of shellfish growing areas following sewage spills. Further studies are required to establish the potential association between the virus dilution factor and the presence of NoV in oysters, as it has been demonstrated that the accumulation of NoV in oysters can vary according to genotype (118, 239) and oyster species (120).

*E. coli* and faecal coliforms are considered useful indicator organisms to assess shellfish safety in shellfish growing areas immediately following faecal contamination events (148). In this study, which generated data from five days after the pollution event, the changes in *E. coli* concentration in oysters at each site over time were not significant. Some sites showed sporadically high levels of *E. coli* (e.g.  $\text{MPN} \geq 18,000$  *E. coli* / 100g shellfish). These ‘spikes’ may have been related to the run-off after both rainfall events notably from cattle production near site 3 and the presence of feral and native animals in the catchment (240). This reinforces existing evidence of the lack of utility of *E. coli* as an indicator for viral contamination beyond the first few days following a faecal contamination event (148) .

The findings of this study demonstrate that NoV GII can persist within SROs for up to six weeks and that NoV GII can be dispersed over many kilometres within estuaries and contaminate oyster stocks. The NoV GII reduction rate over time observed in this study was similar to that observed in other studies. The results of our study will be helpful to local health authorities in defining closure areas and times for commercial oyster production areas following sewage spills. Further data should be collected on spatial and temporal distribution of NoV in oysters following sewage overflows under a broader range of conditions e.g. temperature, salinity, water flow and dilution factors, to improve prediction of elimination timeframes and spatially impacted areas.





# Chapter 6: Evaluation of risk management of Australian oyster harvest areas for the protection of oyster consumers from NoV illness

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## 6.1 Introduction

This chapter investigates the likelihood of NoV contamination of Australian oyster harvest areas and whether the current risk management system for the oyster industry is able to reliably protect consumers of Australian oysters against NoV illness.

Given the paucity of directly relevant data to enable a fully quantitative risk assessment, the approach taken is to use available knowledge and qualitative and semi-quantitative risk characterization approaches (241) to:

- i) use current knowledge to review the pathways and probabilities of contamination of Australian commercially-grown oysters with NoV,
- ii) explore the consequences of theoretical contamination events to gauge whether current risk management systems reliably protect Australian oyster consumers from NoV illness, and
- iii) discuss further risk management options for reducing the possibility of undetected NoV contamination in oyster harvest areas.

## 6.2 Review of potential sources of NoV contamination in Australian oyster harvest areas

Sanitary surveys for six oyster harvest areas were selected for review. Four, of the areas NSW1, TAS1, TAS2 and SA2, have been surveyed for the presence of NoV *see* Chapter 4, and were considered by the shellfish authorities in their respective States as the most compromised with respect to the potential for human faecal contamination as identified from the shoreline surveys. The harvest areas were chosen so as to develop a conservative (*i.e.* closer to “worst case”) assessment. NSW 2 was intensively surveyed for NoV as part of an investigation into potential

NoV contamination of a growing area (see Chapter 3). NoV has been detected in oysters from harvest areas NSW1 (*see* S.4.4.1), and NSW2 (*see* S.3.4.1). Table 1.1 provides numbers of classified harvest areas in each State, with SA having the most “approved” harvest areas and NSW the most “restricted conditional” harvest areas. All areas were classified according to the criteria contained in the ASQAP Operations Manual (145) (Table 4.1).

Potential sources of NoV contamination were gathered from sanitary surveys provided by State Shellfish Authorities from the States of Tasmania (‘TAS’), South Australia (‘SA’) and New South Wales (‘NSW’) and are listed in Table 6.1. The potential sources of NoV contamination of highest risk in these surveys are discussed in S6.2.1 On-site sewage management systems, S6.2.2 Sewage treatment plants and S6.2.3 Marine vessels.

**Table 6.1:** Potential sources of NoV contamination as observed in six selected sanitary surveys.

Harvest area	Sanitary survey classification	Popn.	Sewage Treatment Plant (STP)	On-site Sewage Management System (OSMS)	Stormwater Drains (SWD)	Marine vessels	Other
TAS 1*	Approved conditional	2,050	3  1 Sewage outfall 0.5km from harvest area	Yes	25	5 fishing processors  84 vessels moored 5km from growing area	3 creeks
TAS 2*	Approved conditional	1050	No	Yes	4	40 permanent	swimming
NSW 1*	Restricted conditional	1,200,000	3 STP  60 discharge points		53	65 registered (commercial)  1 pump-out facility  40 recreational	
NSW 2#	Restricted conditional closed	3000	2 STP 16 pump stations, 5 classified as “high risk”	77 high risk, 200 medium risk	16, some discharge directly into harvest area: classified as “significant risk”	Recreational fishing, sailing	swimming
SA 1	Approved	100  1.5 km closure zone	No	All OSMS upgraded ~2005 §	13  1 wastewater mainly oyster washing waste	Fishing boats and recreational  70 berth marina (7km away)	swimming
SA 2*	Approved conditional	350-2000	No	Yes	9	Fishing boats and recreational	swimming1 creek

\* Harvest areas surveyed for NoV in S.4.3, # harvest area surveyed for NoV in S.3.3, § (C. Wilkinson, SASQAP 2015 *pers.comm*)

### 6.2.1 On-site sewage management systems

On-site sewage management systems (OSMS) are systems that collect and treat wastewater generated on the premises which are not connected to a reticulated sewerage system. They are typically household septic tanks and aerated wastewater treatment systems installed by the landowner. The septic holding tank is buried into the ground and bacteria generated in the waste help to break the waste down. There are three layers formed from the waste as it is treated within the holding tank; light waste or scum floating on top of a liquid layer (effluent), and the heavier sludge on the bottom. The effluent leaves the tank through an outlet pipe as new waste enters. The effluent contains dissolved pollutants (salts/phosphorous/nitrogen) and potentially pathogens. The effluent is discharged directly into soil, below ground level, through pipes and trenches where the soil components slowly break down the pollutants and pathogens. In areas where the soil is unsuitable for effluent disposal, special absorption fields may be constructed or the effluent stored in a holding tank for collection and disposal off-site. Routine removal of the sludge needs to be done every 3-5 years to prevent buildup of the sludge (242).

In most circumstances, OSMS that are functioning correctly do not pose a risk to shellfish growing areas. However, when these systems are faulty or not maintained there is a risk that human enteric viruses will leach into the growing areas (243-246). Faulty OSMS were identified as potential sources of contamination linked to the Wallis Lake Hepatitis A virus (HAV) outbreak in 1997, when more than 300 people contracted illness, and one person died, due to HAV from contaminated oysters (164). HAV, like NoV, contaminates oysters from inadequately treated human faeces present in the growing water and is similarly robust in the environment and oysters. Audits showed that OSMS adjacent to NSW2 and approved for installation by the local council were faulty (163). The harvest area of NSW2 was closed for direct shellfish harvesting following outbreaks of NoV in 2008, which were linked to shellfish from the area *see* Chapter 3. The local council has since required property owners in the vicinity of NSW2 to upgrade OSMS or to connect to the region's reticulated sewerage system.

In 2000 in NSW it was estimated that 70% of OSMS failed to meet environmental and health protection standards (242). A review of domestic wastewater in NSW (247) was conducted by the NSW Legislative Assembly in 2012. It documented 18 recommendations for improving the management of OSMS in NSW (247). Many of the recommendations are still waiting to be addressed (248). Recommendations such as updating guidelines for OSMS management and the enforceability of those guidelines are currently awaiting review by the Local Government Acts

Taskforce (248, 249). In SA and TAS, home owners with OSMS are legally required to maintain their systems *see* Table 6.2.

The NSWFA estimated that in 2012, 4225 OSMS were located in the catchments of shellfish harvest areas (248). Of 74 harvest areas, 72 have OSMS in the catchment (A. Zammit, NSWFA, 2015 *pers. comm.*). In TAS, almost all the harvest areas have OSMS in the catchment (A. Turnbull, previous Manager of TASQAP, 2015 *pers. comm.*). A report on the ecological condition of the D'Entrecasteaux Channel waterway in TAS describes septic tank “hot spots” of high numbers, and failures due to older OSMS, high-density dwellings and poor soils situated around the waterway which holds several shellfish growing areas (250). TSQAP are permitted to inspect OSMS in harvest areas that have the potential to pollute the shellfish (Table 6.2) and direct the harvesting according to risk. In June 2015, the TAS Director of Health showed concern for run-off from failing OSMS and included in a Public Health Alert a warning “not to eat wild shellfish harvested near areas where septic tanks (OSMS) may be failing” (251). In SA, OSMS are found in all harvest area catchments. Only one harvest area has OSMS that could potentially contaminate the harvest area, SA2, which is classified “conditionally approved” (C. Wilkinson, SASQAP, 2015 *pers. comm.*). In the other SA harvest area reviewed here, SA1, approximately 20,000 to 30,000 people flocked to the townships to experience the total eclipse of the sun in 2002 (252), but no increase in faecal indicators were detected in the harvest areas (C. Wilkinson, SASQAP, 2015 *pers. comm.*). The OSMS in SA1 were upgraded in or around 2005.

On enquiring with local councils responsible for managing OSMS identified in the sanitary surveys in the TAS2, SA1 and SA2 harvest areas, none of the councils inspected OSMS routinely. Clarence City Council is responsible for OSMS management in the TAS 2 area, and inspects OSMS at the time of sale of the property (SA1, T. Theodosiou, Manager Environmental Services, Ceduna Council 2015 *pers. comm.*, SA2 L. Blakker, Manager Development & Environmental Services, District of Lower Eyre Peninsula, 2014 *pers. comm.*, TAS2, S. Street, EHO, Clarence City Council 2015 *pers. comm.*). The legal requirement is to inspect on installation of the OSMS. Table 6.2 provides an overview of the regulations and who is permitted to inspect in NSW, TAS and SA.

**Table 6.2.** Regulations governing OSMS in NSW, TAS and SA.

	NSW	TAS	SA
Regulations	<i>On-site Sewage Management for Single Households</i> 1998 or the “Silver Book” (253)	Use AS/NZ Standards 1547:2000 <i>On-site domestic wastewater management</i> . 1546.1 <i>Septic tanks</i> . 1546.2 <i>Water composting toilets</i> 1546.3 AWTs as specified in TAS Plumbing Code 2006.  Local councils responsible for OSMS complying with code.	Public and Environmental Health (waste control) Regulations 2010. Waste control Systems-Standards for the construction installation and operation of septic tank systems in South Australia 1995 Non compliance is an offence
Local Council Environmental Health Officers permitted to inspect OSMS	Yes	Yes	Yes
Shellfish authority permitted to inspect OSMS	No	Yes	Yes

The NSWFA are not legally permitted to inspect OSMS (*see* Table 6.2). They work closely with local government to ensure that OSMS performance is monitored. Annual reviews of harvest areas that assess potential pollution risks are used to implement a programme of constant incremental improvement for the harvest areas (A. Zammit, NSWFA 2015 *pers. comm.*). SASQAP meets annually with EHO from local councils to discuss potential issues concerning oyster growing areas within their jurisdiction (C. Wilkinson, SASQAP 2014 *pers. comm.*).

Regulation of operating OSMS and maintenance schedules by local councils can ensure that OSMS that have the potential to impact on harvest areas, are functioning appropriately and therefore reduce the risk of NoV reaching oyster harvest areas. The Greater Lakes Council (NSW), which includes Wallis Lake oyster harvest area and catchment, has chosen to regulate OSMS and refers to the NSW Local Government Act 1993, section 68, Part C No. 5 for the authority to do so. Each OSMS owner is required to submit a geotechnical report with the application for OSMS installation. Licensed operators are to be used for installation, inspections and maintenance. Each OSMS owner must be approved to operate, which requires regular

renewal and ongoing council inspections to ensure that a system continues to function properly over its lifetime (see e.g. <http://www.greatlakes.nsw.gov.au/Home>). In a submission to the NSW Enquiry into the Regulation of Domestic Wastewater (248), the Eurobodalla Shire Council recorded that the non-compliance rate of OSMS had dropped from 75% to 15% since the start of the Council OSMS monitoring programme in 1999. The NSW DPI in 2011 estimated that with stricter regulation of OSMS the oyster industry in that State could benefit up to \$900,000 per annum (an increase of 17%) in productivity state-wide (254).

Sanitary survey classifications of harvest areas consider the risk from OSMS, however there is an assumption that the OSMS are functioning according to their specifications (145). It is clear that unless local councils regulate OSMS that pose a risk of contaminating harvest areas there is a high probability that oysters will become contaminated with NoV.

In 2013, Hay, McCoubrey and Zammit (255) studied outbreaks of NoV in shellfish in Australia and New Zealand to identify key factors that “result in failure to prevent norovirus contamination of oysters in growing areas and the resultant norovirus outbreaks in consumers”. Several recommendations from this report, and pertinent to Table 6.1 include:

- i) There should be an initiative to improve linkages between Councils and Food Authorities—joint training initiatives/memoranda of understanding *etc.*
- ii) The development of procedures/templates to use in the event of a suspected viral illness outbreak implicating shellfish growing areas
- iii) Initiatives to increase technical competence of Council and Food Authority officers in assessing the suitability and efficacy of STP and OSMS through targeted training initiatives.

According to Zammit (NSWFA 2015, *pers. comm.*) NSWFA has responded to these recommendations by:

- i) Conducting joint pollution tracking studies (Faecal sterol studies and dye testing of OSMS) with councils. Those projects have improved relationships between the shellfish sanitation programme and the council/water utility and have served to raise the awareness of shellfish safety issues within councils
- ii) Preparing a template for recording sewage overflows. This ensures that information (*eg.* volume, time, source of overflow), is recorded at the time of notification

- iii) Conducting several targeted training initiatives: a joint dye USFDA/NSW study of a shellfish harvest area, was conducted in 2013
- iv) NSWFA shellfish safety managers are sent attend the International Conference on Molluscan Shellfish Safety (ICMSS)

SASQAP has responded to the review by organizing annual meetings with EHO from local councils to discuss potential issues concerning oyster growing areas within their jurisdiction (C. Wilkinson, SASQAP 2014 *pers. comm.*).

## **6.2.2 Sewage Treatment Plants**

### **6.2.2.1 Influent and effluent**

NoV can be found in untreated sewage influent at concentrations up to  $10^9$  genome copies/L depending on the size of the population the STP services, the time of year and whether there is a new epidemic NoV variant infecting the population (104). The larger the population serviced by the STP the more likely that NoV will be present in influent all year round (124). According to Hewitt *et al.* (256) the influent for small STP (population <1100 – 4000) tends to have variable virus concentrations with sporadic spikes in both the influent and effluent. Their study of adenovirus, enterovirus and NoV in influent and effluent from STP of different sizes, showed that culturable adenovirus and enterovirus were present in the effluent. This suggested that a proportion of NoV detected by PCR in the same effluent could be infectious. Others have also shown that some faecal indicators, such as F+RNA bacteriophage and *E. coli* which are more environmentally fragile than NoV, can survive sewage treatment (160). NoV removal rates vary between the types of treatment plants and their processes. Most STP do not remove all NoV with the treatment of sewage (257). In very general terms, a primary STP reduces NoV concentrations by approximately 1-2  $\log_{10}$ /L, secondary STPs can reduce NoV by 2-3  $\log_{10}$ /L and tertiary STPs will reduce NoV by 3-6  $\log_{10}$ /L. A membrane bioreactor tertiary STP was shown to reduce NoV by 6  $\log_{10}$ /L from influent containing 9  $\log_{10}$  of NoV/L, leaving 3  $\log_{10}$ /L of virus in the effluent (165). It was not reported how much of this virus is viable. Oysters placed next to STP outflows have taken up NoV from the effluent (124, 166, 212, 239). Furthermore, oysters placed up to 10 km from an STP outflow accumulated NoV (159, 166). Preliminary investigations by Campos *et al.* (258) have shown that UV treatment of effluent may be effective in reducing NoV concentrations.



#### 6.2.2.2 Overflows

Overflows from STP can cause raw or partially treated sewage to flow into oyster growing areas. In the case of dry weather sewage overflows, which arise from events other than rainfall and that nonetheless have the potential to contaminate harvest areas, notifications of the overflow to the EPA and shellfish authorities are required in all States (*see* Table 6.3). Such events or factors include:

- i) Sewer blockages, which are one of the most common causes of dry-weather sewer overflows and are usually caused by tree limbs penetrating the sewer pipes, and
- ii) Pump stations, when the storage volume for the pump station is exceeded including due to system growth (increased populations).
- iii) Equipment malfunctions, damage, rising main burst, age of the infrastructure
- iv) Power failures
- v) Major industrial discharges: large industries can have large waste retention tanks, from which the waste can be discharged to the sewer quickly. If multiple discharges occur together, the system may overflow (259).

Sewage overflows also commonly occur as a result of significant rainfall or flooding (*see* Chapter 5). In Australia most sewerage systems do not carry stormwater. Wet weather overflows can occur when rainwater enters sewers either from rain seeping through soil into leaky sewers, from illegally-connected stormwater pipes, or from broken property drains. When the capacity of the sewer is exceeded, overflows from the overflow structure points occur. Untreated sewage can flow into the oyster harvest areas with the additional rain water. This is because many old STP have storage capacities that are too small to contain sewage in high rainfall events. Regulations for STP operations often exempt them from notifying shellfish authorities and the EPA about wet weather overflows associated with high rainfall. Operators of new STPs are penalized if wet (or dry) weather overflows occur.

Harvest areas are routinely closed in response to significant levels of rainfall, increased river flow and decreased salinity in these situations. Oysters are known to recover from large reductions in salinity and return to normal filtration rates within six hours (238) however, and high levels of NoV have been detected in oysters within two days of the start of a flooding event (237) and five days after a pump station sewage overflow, down a river with increased river flow (*see* Chapter 5).

**Table 6.3:** Government regulations requiring notifications of sewage spills impacting harvest areas to State shellfish authorities.

State	Notification to Shellfish authority	Regulations associated with notification requirement
NSW	Yes	NSW Protection of the Environment Legislation Amendment Act 2011 and the NSW Protection of the Environment Operations Act 1997
TAS	Yes	Tasmanian Environmental Management and Pollution Control Act 1994
SA	Yes	SA EPA Act 1993

Table 6.4 lists the sewage overflows reported to the respective state Environmental Protection Agencies (EPA) and the notifications to Shellfish Authorities of overflows that had apparent potential to reach shellfish harvest areas indicating apparent under-reporting. It is uncertain whether sewage overflows are actually under-reported to the Shellfish Authorities because it is not reported whether some of the overflows were considered not to have had the potential to reach harvest areas.

**Table 6.4:** Sewage overflows occurring in the six harvest areas investigated for this profile and the notifications recorded by the respective shellfish authorities

State	State averaged sewer overflows/100km of sewer main	Total sewage overflows per year Note: overflows may not have reached harvest area.	Notifications to Shellfish authority
NSW 1	State average of 5 dry weather overflows/STP  Sewer overflows reported to environmental regulator (per 100 km of sewer main)  2010-2011 1.23  2012-2013 0.32 (260)	STP A EPA annual return (261):  2009-12: <b>76</b> overflows  STP B EPA annual return (262):  2009-12: <b>12</b> overflows, 1 release of partially treated sewage	None
NSW 2	As above	No overflows noted in annual returns	2010-13  Two notifications
TAS 1	Sewer overflows per 100km of sewer main  2010-2011 5.7  2011 -2012 3 (263)	STP was 1 of 35 STPs (owned by a single authority) all of which had 107 overflows for 2010-2012	2010-12  Five due to flood events,  1 pump station failure
TAS 2		OSMS only	None
SA 1		OSMS only	None
SA 2		OSMS only	None

#Includes pump station overflows, overflows due to power failures and minor overflows due to blocked drains. \*Shellfish Authorities in all States, are required to be notified if there is potential for sewage overflows to reach shellfish growing areas.

### 6.2.3 Marine vessels

Sewage disposal from commercial marine vessels is regulated by environment protection regulations. Sewage pump-out facilities for emptying on-board toilets are provided in some council areas where there are high numbers of boat users. A pump-out facility is mentioned as being provided in one (NSW 1) of the sanitary surveys. However, there is also a possibility of NoV contamination from small vessels in oyster harvest areas. This is because smaller vessels are less likely to have toilet facilities with effluent retention tanks. There have been several notable NoV contaminations of oyster harvest areas in the USA, because oyster workers discharged their sewage directly into the water due to a lack of toilet facilities on board the vessel, and in Canada when oyster workers expelled vomitus directly into the harvest area (161, 162). A USA NoV outbreak was linked to oysters from a remote harvest area in Louisiana where an estimated 4.6 million oysters were believed to have been contaminated by a single worker with diarrhoea (264). The oysters had acceptable concentrations of faecal coliforms as per NSSP requirements (168). It is believed that more than 300,000 people consumed the oysters and up to 186,000 people may have developed symptoms and became ill (264). Houseboats and marine vessels were also implicated in the Wallis Lake oyster-linked outbreak of HAV (164).

Regulations about discharge of sewage from marine vessels into water vary from State to State. Section 25CB(2) (ab) of the Pollution of Waters by Oil and Noxious Substances Act 1987 in TAS states that marine vessels carrying fewer than 15 people must not discharge sewage within 500m of oyster harvest areas or into any intermittently opening or closing lagoon (and also names specifically most oyster harvest areas in the State). In SA, the Code of Practice for Vessel and Facility Management (Marine and Inland Waters) 2008 stipulates that blackwater (untreated or treated sewage) must not be discharged into State waters from marine vessels within three nautical miles from aquaculture leases. In New South Wales under the Protection of the Environment Operations Act 1997 (POEO Act) together with the Marine Pollution Amendment (Waste Discharge and Oil Spill Response Plans) Regulation 2003, the discharge of untreated sewage from vessels is prohibited in navigable waters. Treated sewage is not to be discharged within 500 m of oyster harvest areas. Treatment of sewage needs to be highly effective to reduce potentially viable NoV from reaching the harvest area, *i.e.* even with 99% inactivation 250 mL of  $10^{12}$  per mL still contributes 1% or  $250 \times 10^{10}$  viable NoV virus particles to contaminate oyster harvest areas 500m away. NoV was detected 5.29km downstream in oysters at a concentration of approximately 800 genome copies/g shellfish gut (*see* Chapter 5). This is much more than the minimum infectious dose (ID<sub>50</sub>) of 7 virus particles. Three nautical miles, the

distance required in SA for the discharge of blackwater from a vessel is the equivalent of 5.56km and to place this in context, is just 270m more than the distance that NoV was detected in oysters resulting from the pump station overflow.

Under Australian regulations for discharging sewage from marine vessels, vessels are permitted to enter into the harvest areas up to the limits of the oyster leases. This permits the vessels to within two to ten metres of the lease. It then depends on the goodwill of the people in the vessels and the threat of detection and subsequent monetary penalties to prevent discharge of sewage near the harvest areas.

## 6.3 Extent and effectiveness of current risk management practices

Data from recent outbreaks of NoV illness due to contaminated Australian oysters were collated and are presented in Table 6.5.

**Table 6.5:** Published data of selected outbreaks of NoV illness linked to Australian oysters.

Year of outbreak and harvest area classification	Number of clusters and cases	NoV detected	Cause	Remediation	Duration of harvest area closure	Ref.
(A) NSW 2005 Conditional restricted	No illness recorded.	oysters -adenovirus, enterovirus □	OSMS, small number faulty.	Dye studies confirmed potential sources. OSMS upgraded	Several harvest areas closed for two years	(265-267)
(B) NSW 2008 Conditional restricted	Two clusters, Est.14 cases	Epidemiological link to oysters*	OSMS, leaking sewer pipes, aerated wastewater systems	OSMS upgraded or connected to sewage system	Six years ongoing	(163)
(C) NSW 2012 Conditional restricted	Est. at 32 cases	faeces and oysters faeces-NoV GII.4	Leaking sewer line	Repaired	Three months	(106)
(D) TAS 2013 Approved conditional	Total of 525 cases, TAS 306, VIC 209, NSW 8, QLD 2	faeces and oysters faeces-NoV GII.1	Private sewer pipe leaking underwater.  Fast distribution of product, mislabelling and co-mingling of product at point of sale.	Repaired	Nine months	(97)

□ A. Zammit, 2015 *pers. comm.*, \*Further testing of oysters from the growing area implicated in the outbreaks detected NoV GII and GI S3.2 and S3.4

The predominant causes of the outbreaks listed in Table 6.5 were leaking sewer pipes and malfunctioning or inappropriately sited OSMS in approved and restricted classified harvest areas. It is unlikely that the untreated sewage would have been detected once in the growing waters prior to the outbreaks, unless faecal coliform and NoV testing was performed on oyster samples physically and temporally close to the pollution source (if the pollution emission was continuous). Faecal coliforms and *E. coli* in oysters and growing waters can be reduced within a few days due to elimination and inactivation under tidal and environmental influences (149-152). Also of concern, is the rapid distribution of oysters product of 24-48h from harvest to consumption, which was an issue for the illness outbreak linked to Tasmanian oysters (251) This makes product recall very difficult if a contamination event is detected after harvesting. Therefore, identification and regular inspections of potential sources of untreated sewage to prevent NoV from reaching harvest areas are critical. Several conclusions drawn from Hay *et al.* (255), shown below, are useful in considering the causes of the outbreaks listed in Table 6.5.

### **6.3.1 Conclusions of Hay, McCoubrey and Zammit**

Hay *et al.* (255) (see also S.6.2.1) analysed information concerning growing areas associated with NoV illness outbreaks in New Zealand and Australia and identified the following key factors that resulted in failure to prevent NoV outbreaks:

- i) *E. coli*/faecal coliform indicators fail to consistently predict the risk of presence of enteric virus, placing a reliance on other components of the SQAP to manage that risk.
- ii) Implementation of sanitary survey components failed to protect consumers as a result of insufficient reliable information gathered during the sanitary survey process
- iii) Reliance on other agencies to provide key information about growing areas and catchments and difficulties in obtaining such information.
- iv) Assumptions by Food Authorities about the quality of management of contamination sources by these other authorities i.e. local councils
- v) Barriers to accessing private properties to inspect potential contamination sources
- vi) Lack of environmental information i.e. hydrodynamics of growing area
- vii) SQAP incorporates infrequent detailed sanitary surveys and minimal field observation annually, and assumes little change will occur in the growing area through time.

- viii) Increasing pressures from competing resource uses – urbanization of coastal areas.
- ix) Implementation of existing environmental policies by councils can be very poor, driven by lack of resources, inadequate management systems and lack of technical competence and expertise.
- x) Lack of expertise and uncritical reliance on standards and guidelines in the design of STPs can result in systems that are inadequately designed to prevent viral contamination of growing areas.
- xi) Failure to manage risk of sources of viral contamination previously implicated in NoV illness events.
- xii) Inadequately detailed documentation of investigations
- xiii) Failure to institute and sustain management plans to ensure contamination issues are not repeated

From Hay *et al.* (255) it is clear that some local Councils responsible for waste-water management in areas where outbreaks have occurred, have failed to improve their management of pollution risks to minimize the potential for further outbreaks. Conversely, some councils responsible for impacted harvest areas have been extremely active in remediating septic and sewage systems. For example, local councils have invested considerable efforts in remediating waterways and catchments where illness outbreaks were linked to harvest areas and the pollution sources were not directly identified, *i.e.* such as for Kalang River and Wallis Lake (M. Hunter, EHO Coordinator, Great Lakes Council 2014 *pers. comm.*) (268, 269). Great Lakes Council successes can also be seen in the River Health Card (270), a scheme which rates ecological health of the waterway using clarity and algal levels in the water. The sites in and around Wallis Lake have maintained A to B ratings for the last four years. An A rating places the waterway in the top 20% in the State.

This review of potential NoV contamination sources in oyster harvest area has shown that there are potential sources within the harvest areas that put the Australian oyster consumer at risk of NoV illness. The main sources of potential NoV contamination selected by their causal association with NoV illness outbreaks linked to Australian oysters (Table 6.5) were OSMS, STP and marine vessels. Upon review of current harvest areas it has been determined that these potential sources of NoV still place the consumer of Australian oysters at risk of contracting NoV illness.



## 6.4 Theoretical contamination events

From existing knowledge of NoV excretion and contamination events affecting shellfish harvest areas, it is possible to consider the likely consequences of hypothetical contamination scenarios in Australian harvest areas. Several research findings are relevant when considering these scenarios:

- i) Oysters can accumulate NoV from their growing water over time (this is particularly important when low levels of NoV are in the water (271))
- ii) The accumulation of viruses by oysters varies according to the environmental conditions to which the oysters are exposed. The uptake may also depend on the genogroup and genotype of NoV (118, 119, 271).
- iii) NoV has been detected in oysters within an hour of the water they are growing in being contaminated with NoV (118)
- iv) NoV can be detected in oysters for between six and eight weeks after the contamination event (119, 156) and Chapter 5.
- v) Very low concentrations of NoV in Australian oysters have been associated with illness. Traces of NoV GII were detected in oysters associated with the Northern NSW and TAS outbreaks ( $C_T$  values of 38 and 41 respectively, A. Turnbull SARDI Food Safety 2015, *pers. comm.*). Approximately 100 virus particles per g shellfish gut was associated with an illness outbreak investigated by Le Guyader *et al.* (102). Thebault *et al.* (108) studied French NoV illness outbreaks linked to shellfish, a median  $ID_{50}$  estimated between 1.6 and 7.51 genome copies per oyster was found to cause illness.
- vi) The detection limit for NoV in the same laboratory was estimated at 70 RNA copies/ g shellfish gut (206) and the detection limit for NoV GII.4, achieved for this Thesis was 260 genome copies/ g shellfish gut (*see* Chapter 2).

### 6.4.1 Hypothetical event of single episode of diarrhoea

Consider a single event such as a spill of diarrhoea from a swimmer, or boaters, near to a harvest area. The classification of the harvest areas in the sanitary surveys recognizes the risk of such an event however there appears no way to identify the event if it occurs. *eg.* one swimmer or boat, 500m away from the harvest area releases up to 250 ml of diarrhoea with a concentration of  $10^{12}$  NoV particles per ml, excreting a total of  $2.5 \times 10^{14}$  virus particles. A considerable dilution

would have to occur to reduce the concentration so as not to impact the harvest area. Faecal bacterial indicators may not be detected unless they are sampled within a few days of the contamination because they are eliminated from the oysters more rapidly, and they are less robust in the environment than viruses. Faecal bacteria occur in lower concentrations than NoV in human faeces at up to  $10^7$  per gram (272). Estimates of dilution factors are difficult to make, because of the unknown volumes of estuaries, however if such data are available and combined with tidal flux values and models of the prevailing flow patterns they can give valuable information about the potential to contaminate the oyster harvest areas. For example, the high tide volume of the growing area which includes TAS 1 has been estimated as 21 million  $\text{m}^3$  or  $2.1 \times 10^{10}$  litres. If the single contamination event of 1 cup or 250 mL of NoV-laden diarrhoea were to be distributed evenly in the growing area, the concentration of NoV would be approximately  $2.5 \times 10^{14} / 2.1 \times 10^{10} = 1.1 \times 10^4$  virus particles per litre. The flushing time of this particular area is 5.7 tidal cycles which means that the oysters would be exposed to the contaminated water for more than enough time to take up enough virus to cause illness (118, 215). Oysters can take up NoV within an hour of exposure (118) and filter up to six L/h. Similarly for TAS 2 the high tide volume for the entire growing area was calculated at  $6.9 \times 10^9$  litres which would mean the equivalent of a cup of diarrhoea would be diluted to  $2.5 \times 10^{14} / 6.9 \times 10^9 = 3.6 \times 10^4$  virus particles per litre. This area has a faster flushing time of 1.4 tidal cycles (215), however there would still be ample time for oysters to take up enough virus to cause illness because oysters can take up NoV within an hour of exposure (118).

These are scenarios of very small volumes containing extremely high concentrations of NoV contaminating a growing area and environmental stressors of NoV, such as UV degradation and temperature, (172) have not been considered. The resulting theoretical contamination is, however, possible and realistic, and oysters from the harvest area could cause illness in the consumers.

#### **6.4.2 Event of a sewage overflow into a harvest area**

A spatial and temporal study of NoV in oysters resulting from a pump station sewage overflow into a river was described in detail in Chapter 5. The sewage overflow of 3021 litres contaminated oysters strategically placed downstream in the river. NoV GII was detected in oysters 5.29 km downstream and persisted in oysters closest to the overflow for 42 days. After 42 days, NoV GII was still present in the oysters, 50 m from the pollution source at approximately 600 copies/g shellfish gut. The  $\text{ID}_{50}$  for NoV in oysters is seven (108) and if the virus is viable, then there is clear potential to cause illness. The concentrations of faecal

indicator, *E. coli* did not reflect the presence of NoV in the oysters. The total volume of the estuary is 4267 ML and does not include the impact of tide. The dilution of the sewage overflow into the entire estuary was 1:1415. NoV can be dispersed from sewage overflows over extended distances within estuaries and could potentially expose oyster consumers to risk of viral infection.

### **6.4.3 Probability of detecting NoV in a batch of contaminated oysters**

If sewage contamination is suspected and the affected oysters are tested for NoV, the likelihood of detecting NoV will depend on the concentration of NoV in the oysters and the capacity of the NoV test to detect the NoV. The survey described in Chapter 4, of Australian oyster growing areas considered by State shellfish authorities as the most compromised with respect to the potential for human faecal contamination as identified by shoreline surveys detected NoV GII in 1.7 % (n=120) of samples (213).

The probability of detecting NoV in a batch of oysters using ISO/TS 1526-1:2013 was evaluated (47) at the sensitivity specified. The probability of detecting NoV in a random sample from a batch increased with concentration of NoV in the oysters, i.e., at low concentrations (100 genome copies/g digestive tissue) which has been associated with an outbreak of illness (102) and with a 1% prevalence of NoV in the batch, the probability of identifying a random sample as positive was 4.0%; whereas at 500 genome copies/ g digestive tissue and a 1% prevalence of NoV in the batch, the probability of identifying a positive was 8.9%. The probability of detecting NoV in oysters in a harvest area can be improved by increasing the number of samples and careful selection of sampling sites in areas that are most likely to have been exposed to the contamination. This was demonstrated in Chapter 3 where 167 samples were tested from a growing area that had intermittent contamination and NoV GI was detected in 8.3 %. However, overall, NoV contamination in Australian oysters is rare (1.7%) and generally of low concentration (97, 106, 213) which means that the probability of identifying a positive random sample is low unless a very large number of oysters are tested. This emphasizes the need for management strategies to keep NoV away from oyster harvest areas in order to reduce the risk of illness in the Australian oyster consumer.

## **6.5 Additional risk mitigation strategies to protect the oyster consumer.**

### **6.5.1 Risk mitigation strategies for the prevention of NoV contamination**

A range of strategies are presented below that may reduce the risk of NoV contamination into oyster growing areas.

#### **6.5.1.1 OSMS monitoring**

The Greater Lakes Council (NSW), which includes Wallis Lake oyster harvest area and catchment, is at the forefront for monitoring OSMS at installation and for ongoing operation for any Australian Council. As previously mentioned, the council refers to the NSW Local Government Act 1993, section 68, Part C No. 5 for the authority to regulate OSMS. Licensed operators are to be used for installation, inspections and maintenance. Each OSMS owner must be approved to operate, which requires regular renewal and ongoing council inspections to ensure that a system continues to function properly over its lifetime (*see e.g. <http://www.greatlakes.nsw.gov.au/Home>*). This is different to an approval to install, more commonly utilized by Australian councils, which is not reviewed (by councils) after the system is installed and operating. A NSW Independent Pricing and Regulatory Tribunal (IPART) review of best practice regulatory approaches to OSMS, discusses in detail council revenue policy options to cover the increased costs associated with the management of operational OSMS, (*see Appendix A.6*). The success of regulation of OSMS at reducing the risk of contamination through ensuring compliance of the OSMS function (248) suggests that regulation of OSMS that pose a risk to harvest areas should be undertaken to reduce the risk to shellfish consumers. A certificate of inspection and function for each OSMS that poses a risk of viral contamination to the harvest area to be provided to the Shellfish Authority prior to opening the harvest area is recommended.

#### **6.5.1.2 Preventing pollution from marine vessels**

Discharging sewage from marine vessels is regulated in all States, however 3 nautical miles between a blackwater discharge and shellfish growing areas is not far enough and there is an increased risk of NoV contamination of the shellfish (*see S6.2.3*). It is therefore recommended that the minimum distance away from shellfish growing areas for discharging blackwater in SA of three nautical miles be extended (*see S.6.2.3*).

There is no requirement for small marine vessels to carry an on-board toilet or bucket for sewage in Australia. Low cost portable and disposable toilets (\$100) for onboard toilets are increasingly being used by recreational fishermen (C. Wilkinson SASQAP, June 2015, *pers. comm.*). The Maritime rule 40D of New Zealand and the NSSP (168) require toilet facilities are to be provided for crew on oyster harvesting vessels. It is recommended that ASQAP includes guidance that oyster harvesting boats carry a disposable toilet. It is also recommended that all marine vessels are required to carry a portable toilet or on-board bucket for sewage.

Monetary fines are one way of discouraging the contamination of growing areas with raw sewage discharged from vessels. In NSW, Penalty Notices (273) provides for a monetary fine for dumping sewage.

#### **6.5.1.3 Upgrading of STPs**

An STP with the outflow near the TAS 1 harvest area was upgraded to a tertiary biomembrane STP in 2009, due to effluent from the previous plant not complying with DPIWE 2001 guidelines (274). The new STP was selected by the local council because of the lower life cycle cost, operational flexibility and ease of operation. The higher quality effluent from the plant has reduced the risks to the health of recreational users of the waterway and maximises effluent reuse. The cost of the plant was offset by a Federal grant from the Clean Quality Water Programme (274). This type of STP has been shown to reduce NoV concentrations from the influent by one million-fold when operating at or under capacity. This level of removal is more than 100 fold more effective than most secondary STPs/treatment processes (165). Upgrading STPs that impact oyster growing areas may reduce the concentration of NoV and other human pathogens in the STP effluent and therefore reduce the risk of oysters becoming contaminated with the virus.

#### **6.5.1.4 Dye studies**

Dye studies can improve the predictability of the flow pattern and dilution factors of faecal pollution and be used to reduce harvest area closure times and the size of the area closed for harvesting. Goblick *et al.* (159) described the flow and dilution of an STP outflow using dye and oysters installed in a bay used for oyster culture. Results showed that the levels of indicator microorganisms inversely correlated with increased dilution of the effluent. Oysters accumulated NoV at a dilution of 1:556 and a distance of 5.29 km from the STP outflow site. A dye study conducted by the FDA and NSWFA during 2013 in NSW, followed the dispersal of a sewage overflow enabling prediction of the flow pattern and dilution of the dye – and pollution (275).

The study has contributed to much reduced closure times (A. Zammit, NSWFA 2014 *pers. comm.*)

Mandatory dye studies for all harvest areas could further reduce risk to consumers whilst improving outcomes for shellfish farmers.

#### **6.5.1.5 Environmental management plans**

Environmental management plans created and supported by local councils and communities in NSW for the Brunswick River (276), Kalang River, Tilligerry Creek (268) and Wallis Lake (269) have led to vast improvements in water quality, in these estuaries (270). In the process of remediating their estuaries, councils have remediated or removed polluting sources. As previously mentioned, Wallis Lakes achieved an “ A” rating for its waterway reportcard in 2014 (270), placing the waterway in the top 20% in the state for ecological health. Despite comments from Hay *et al.* (12) about the lack of adherence to environmental plans, it is evident that when such plans are embraced by councils and their communities, cleaner water and environs have been achieved. Markers of river or estuary health can be presented in River/ Estuary reports and can be used to advertise the waterway, attract funding and increase tourism. In 2004, Great Lakes Council was awarded the Theiss International Riverprize (277) for best practice in river and catchment management and environmental repair in Australia and in 2010 the Byron Shire Council won a United Nations Association of Australia’s *Local Government Award for Excellence in Overall Environmental Management* for the Brunswick River Estuary Management Plan and Sewerage Augmentation Scheme (278, 279).

#### **6.5.1.6 Education and communication**

Results from this risk profile and the recent review of outbreaks by Hay *et al.* (255) suggest that there is a need for education about NoV and improved communication between STP operators, local council Environmental Health Officers (EHO), shellfish authorities and shellfish growers. It may be possible to incorporate an automated system for recognition of the occurrence of spills and to initiate action into these communications. Improving EHO and waste water (STP) operator knowledge about NoV and the potential for contamination of shellfish growing areas could be achieved through:

- i) Presentations from shellfish authorities at State and national EHO conferences (*see* <http://www.eh.org.au/>)

- ii) Presentations by shellfish authorities to STP operators at State and national industry conferences. Water Industry Operators Association (WIOA) (*see* [www.WIOA.org.au](http://www.WIOA.org.au))
- iii) Articles by shellfish authorities or experts in the field, for publication in industry journals or as stand-alone publications
- iv) Provision of links on EHO and WIOA websites to Safefish. Safefish is a website providing documents about NoV and contamination of shellfish (280)

The adoption of a joint pollution tracking studies by the NSWFA with councils and water utility operators, as previously mentioned (*see* S.6.3.1) has raised awareness of shellfish safety issues within participating councils and water utilities (A. Zammit, NSWFA *pers. comm.* 2015).

SASQAP meets annually with EHO from local councils to discuss potential issues concerning oyster growing areas within their jurisdiction (C. Wilkinson, SASQAP. 2014 *pers. comm.*).

Information from research in the fields of NoV and shellfish is rapidly expanding and one way for shellfish authorities to keep up to date could be to attend international conferences such as the biennial International Molluscan Shellfish Safety Conference (ICMSS). An annual workshop focussing on pathways to improve water quality is recommended for EHO and shellfish authorities. Funding could be sought from the Fisheries Research and Development Corporation.

## **6.5.2 Risk mitigation strategies for the early detection of NoV contamination**

Strategies should be in place for early detection of potential NoV contamination events to ensure a rapid response for preventing illness in the oyster consumer.

### **6.5.2.1 Compliance/ batch testing for NoV**

The prevalence of NoV in oysters varies by region, with reports of 9 % in Japan (175), 56.8% in UK (176), 14 % in France (281) and 31 % in Ireland (282) of shellfish samples tested from commercial areas. These data have led to the proposal that compliance limits be imposed to protect consumers of EU shellfish (140). The average percentage of samples that would fail under various circumstances has been estimated by EFSA (140)A maximum of 100 copies/g would have failed 65.6% (UK), 83.3% (Ireland) and 33.6% (France) of shellfish samples during January to March 2010 (271). The prevalence of NoV in Australian oyster growing areas has been reported as 1.7% (213) using a comparable detection method to the aforementioned countries and is considerably lower than the prevalence reported for the EU countries to date. Batch testing and incorporating enough samples to detect the rare occurrence of NoV would be prohibitively costly *see* (S.6.4.3).

In Australia, due to the low frequencies of contamination, effective batch testing would be expensive and while it would certainly identify grossly contaminated product, it would not be able to reliably detect contamination levels relevant to the enhanced protection of public health. To explain, for simplicity assume that the ID<sub>50</sub> for NoV is approximately 20 viral particles in histo-blood group secretor status SE<sup>+</sup> cases (49), and that a typical serving of oysters is 100g of oyster flesh.

In a 100g sample samples of oysters, there are approximately 6 oysters and each with ~1g of gut. The detection method employed in this Thesis can detect as few as 7 NoV genomes in 0.025g oyster gut. Thus, at a simple, deterministic level, if we assume homogenous distribution of virions, a negative result infers only that there are likely to be less than 7 virions per 0.025g, but there are 6 g of gut tissue per 100g serve. Thus, as many as virions 1440 could be present in a serve and escape reliable detection, *i.e.*, vastly in excess of the ID<sub>50</sub>. To protect public health, a level of contamination that results in a probability of illness of <1% per serve may be considered an ‘appropriate level of protection’, given that consumers in developed countries choose to eat raw oysters for enjoyment, rather than nutritional need. In essence, this means that an acceptable level of contamination is, on average, <2 NoV per 1 kg (*i.e.*, <20 virions in 1% of 100g serves).

In the methods used in this Thesis a single test contains 0.1g of oyster. At the maximum tolerable level of one NoV per 500 g, only one in 5000 samples of 0.1g of oyster could be tolerated to contain a NoV particle.

Using the simple binomial distribution it can be shown that to assure, with 95% confidence, that the contamination is at or below two NoV per kg, a level assumed for this example to be commensurate with an “acceptable” risk to public health, would require testing 14,978 samples of 0.1 g per batch, all of which would have to be negative. Clearly, testing cannot be used routinely to assure product safety.

The statistics of presence/absence testing are well understood and probabilities of detection, as a function of actual proportion of faulty batches, can be calculated using the binomial (or, more correctly, the hypergeometric) distribution (283). In general, for batches with less than 5% units being defective, impractically large numbers of samples (*e.g.* > 60) need to be tested. Testing may be useful for routine monitoring over time, or to identify grossly contaminated batches, but it impractical to assess an acceptable level of protection on a routine basis. This is why preventative methods are more cost-effective. Testing frequency should be commensurate with the risk associated with the growing area.



#### **6.5.2.2 Bacteriophage testing**

F+RNA bacteriophage testing is used by the USFDA (168) as an additional indicator of untreated sewage contamination in shellfish growing areas. ESR (NZ) found that bacteriophage results did not correlate clearly with faecal contamination except in an area where shellfish were growing in close proximity to a sewage outfall (166) and Goblick *et al.* (159) found a maximum of 2296 pfu/100g Eastern oysters placed in the effluent plume 50m from a secondary wastewater treatment plant which serviced 200,000 people in Mobile Bay, USA. Further studies are recommended for Australian harvest areas to investigate the potential of bacteriophage as an alternative indicator for untreated sewage contamination.

#### **6.5.2.3 Rapid reporting of sewage overflows**

With the advent of mobile phones it is now possible for shellfish authorities and oyster growers to be alerted of sewage overflows rapidly thus reducing the risk of NoV contaminated oysters reaching the consumer. It is recommended that the State shellfish authorities utilize mobile phone incident alert systems.

#### **6.5.2.4 Accurate recording of sewage overflows**

Details of sewage overflows in NSW growing areas are recorded by NSWFA on a specific form which ensures that information about the size of the overflow, time, date, location, environmental conditions *etc.* is recorded. This information helps to coordinate sampling from the impacted harvest area, the size of the area to be closed and closure times (A. Zammit, NSWFA, 2015 *pers. comm.*).

Guidance for managing sewage overflows in harvest areas *i.e.* closure times and delineation of closure areas that have been affected by the spill are not in the ASQAP but would be helpful and ensure a consistent response to sewage spills, if included in ASQAP. Research conducted in Chapter 5, mapped NoV GII in oysters, that were distributed as far as 5.29 km from the source of pollution and were detected in oysters for as long as 42 days after the pollution event. A reduction rate of 8.5% of NoV from the oysters per day was observed and was found to be comparable with other NoV elimination studies. None of the studies in this Thesis found correlation of the presence of NoV with *E.coli*, the currently used bacterial indicator for contamination of shellfish. The data collected for this Thesis should be used to inform the development of policies on closure times and distances. More studies are recommended to investigate closure times and distances under different conditions.

#### **6.5.2.5 Systems to enable more rapid response times**

##### **6.5.2.5.1 Sense T**

Sense T ([www.sense-t.org.au](http://www.sense-t.org.au)) is a University of Tasmania initiative with the Intelligent Sensing and Systems Laboratory, ICT Centre, Commonwealth Scientific Research Organization, TSQAP and other partners. It is a system that can gather environmental data such as salinity and temperature and potentially data such as oyster function (heart beat/ stress *etc.*). The system will be used to monitor TAS growing areas in real time and allow for a faster response to triggers for opening and closing oyster harvesting areas. It is recommended that this and other alternative systems that will speed up the response times to deal with contamination events, are investigated and supported in NSW and SA.

##### **6.5.2.4.2 OceanWatch EMS**

OceanWatch NSW has instigated an environmental management system (EMS) for oyster growing areas in NSW. To date 20 out of 33 growing areas (60%) have joined the system. EMS encourages the growers to improve their estuaries by identifying and managing the risks for the oyster growing area environment. A major part of the EMS is dealing with risks of sewage contamination. The system encourages a proactive approach by the oyster industry to identify pollution risks and remediate before contamination eventuates. <http://www.oceanwatch.org.au/our-work/ems-nsw-oysters/> Development of the EMS for oyster growing areas in other States is recommended.

##### **6.5.2.4.3 Local Management Committee**

The NSWFA appoints a local shellfish committee to help the Authority administer the local programme (284). The committee enables the oyster industry to take responsibility for their own growing areas, conduct risk assessments of potential pollution sources and have a proactive approach in harvest area closures due to sewage contamination, *e.g.* using the approaches and technologies outlined above. The formation of local management committees for each growing area is recommended for all States with oyster growing areas.

## 6.6 Recommendations and Summary

There are an estimated 1 -2 outbreaks of illness annually related to NoV in Australian oysters (15). However, NoV contamination of Australian oyster growing areas is rare, with NoV detected in 1.7% of oyster samples (n =120) from Australian growing areas (Chapter 4). It is not feasible to end-product test all Australian oysters to ensure the safety of the consumer when the overall NoV prevalence is very low. Oysters must be harvested from areas free from NoV contamination. This means infectious NoV should be precluded from harvest areas through a range of management activities such as recommended below:

- i) Regulation of OSMS, by local councils to ensure OSMS function. Issuing of certificates of inspection and function for OSMS that pose a risk of contamination to harvest areas. Harvest areas to be closed for harvesting until Shellfish Authorities are provided with all Certificates for OSMS that pose a risk to the harvest areas.
- ii) Upgrade STPs to reduce NoV (and other human pathogen) concentrations in effluent that flows into oyster growing areas and conduct studies to ensure that harvest areas are not within the effluent path.
- iii) Mandate dye studies of STP outflows and other potential sources of sewage contamination into oyster harvest areas for all harvest areas with potential sources of sewage contamination. This would allow for better prediction of the flow of sewage spills which can, in turn reduce closure times and the size of the area impacted by the closure.
- iv) Conduct workshops/conference presentations about viral contamination of harvest areas for EHO/water utilities and shellfish authorities.
- v) ASQAP to include guidance for local management committees to be formed for each oyster growing area.
- vi) ASQAP to include guidance that oyster harvesting boats carry a disposable toilet
- vii) All small marine vessels to be required to carry a portable toilet or on-board bucket for sewage.

- viii) Increase the three nautical mile minimum distance away from shellfish growing areas for discharging blackwater in SA.
- ix) Extend EMS and develop local environmental management plans to advance the remediation of potential pollution sources in TAS and SA.
- x) Investigate alternative mechanical systems *i.e.* mobile notifications/ SENSE-T, that will speed up the response times to deal with contamination events as they occur.
- xi) Further studies on the spatial and temporal distribution of NoV in oysters following NoV contamination to determine more accurate closure times and distances for harvest areas.

Australian oyster consumers are currently at low, but significant risk of NoV illness from domestic oysters. This evaluation of the risk management of Australian oyster harvest areas has made recommendations additional strategies to reduce the risk of NoV illness to oyster consumers.

# Chapter 7: Discussion and future directions

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## 7.1 Summary and Conclusions

NoV contamination of oysters may be considered a ‘wicked problem’. NoV is a pathogen that is hard to detect, is environmentally stable, takes only a few virions to cause illness and even the best shellfish safety programme may not be able to prevent contamination of a harvest area. Therefore management strategies for the prevention and minimisation of NoV contamination and its early detection if it occurs in the harvest areas, are an important way to reduce the risk to oyster consumers. The results and analyses presented in this Thesis significantly contribute to an informed strategy for minimising the risk of NoV illness from the consumption of Australian oysters. This has been achieved by

- i) Validation of an internationally accepted testing method for detection of NoV in Australian oysters.
- ii) Developing and trialling a sampling programme for investigating NoV contamination in Australian oysters that is able to detect low concentrations and low occurrence of NoV.
- iii) Using these approaches to estimate the prevalence of NoV in Australian oysters.
- iv) Investigating the spatial and temporal spread of NoV in oysters following a sewage overflow in a river estuary and demonstrating that NoV GII can persist within SROs for up to six weeks and can be dispersed over many kilometres within estuaries to contaminate oyster stocks.
- v) Estimating the NoV GII reduction rate and showing that it is consistent with estimates from other growing areas around the world.
- xii) Evaluating the efficacy of current risk management strategies for Australian oyster harvest areas and protection of oyster consumers from NoV illness.
- xiii) Proposing additional and alternative management strategies for preventing and minimising the risk of NoV illness to the oyster consumer.

Australian oysters have been linked to NoV illness outbreaks (15). The contamination can occur when NoV from human excrement (faeces or vomit) flows into oyster growing areas. The growing areas are monitored and managed according to ASQAP using bacterial faecal indicators (145). These indicators are considered useful immediately following faecal contamination events but can decrease within a few days due to elimination and inactivation under tidal and environmental influences, rendering them unreliable for assessment of potential viral contamination. Prior to the studies described in this Thesis, minimal data existed on the occurrence of NoV in Australian oysters. This was largely due to the expense involved in testing oysters for the virus and the lack of the capacity for routine testing in Australia. Consequently oyster samples were sent to New Zealand for NoV testing. Impending international policies on NoV testing of shellfish added impetus for development of capacity for NoV testing of Australian shellfish in Australia.

In this Thesis, an internationally acceptable method for detecting NoV in shellfish (129), determined to be both accurate and sensitive, was validated for use under Australian conditions by the candidate (*see* Chapter 2).

There is a lack of consensus worldwide about sampling regimes for the detection of NoV in shellfish. To address this, a study was conducted of NoV contamination of oysters in a river estuary. Oysters from the estuary had been epidemiologically associated with two small outbreaks of NoV illness and NoV had been detected in oysters from the harvest area for up to three months after the outbreak. The study described in Chapter 3 provides an example of a sampling programme for investigating NoV contamination of a growing area where NoV concentrations and occurrence in oysters are low. Pre-existing data and information gathered by consultation with those familiar with the harvest area, its environs and SRO, were important in the selection of potentially impacted sampling sites.

The intensity of the sampling programme, which consisted of five collection events during routine and adverse environmental conditions, with five samples collected from each of seven sites over seven months, enabled the detection of NoV GI in the SRO despite the occurrence and concentration of NoV being low. The study showed variation in NoV uptake between SRO samples taken from within the same site on the same day and revealed ongoing sewage contamination.

*E. coli* was monitored in the oysters sampled in each study reported in this Thesis to enable comparison with NoV detections. The presence or absence of NoV in oyster samples was not found to be related to the concentrations of *E. coli*.

A study to determine the prevalence of NoV in Australian oysters presented in Chapter 4, used two geographically-distinct oyster-growing areas from each of three Australian States. The sites were sampled on 4 occasions during 2010 and 2011. The sites selected were considered by State shellfish authorities to be the most compromised with respect to the potential for human faecal contamination. Oysters were tested for NoV GI, GII and *E. coli* and each site was sampled on four occasions between 2010 and 2011. NoV GII was detected in two of 120 (1.7 %) samples while NoV GI was not detected. One of the samples with NoV was cloned and identified by gene sequence as GII.3. Five of 120 (4.2 %) samples were found to exceed the guidance level of 230 *E. coli* per 100g of shellfish but these samples did not contain detectable levels of NoV. The apparently low prevalence of NoV in oysters from Australian growing areas supported epidemiological data (15) that suggested NoV contamination of Australian commercial oysters is rare.

Information about the persistence of NoV in SRO was not available prior to the studies described in this Thesis. Chapter 5 presented the first study of the spatial and temporal distribution of NoV in SRO following a sewage overflow down a river estuary.

The spatial and temporal distribution of NoV and *E. coli* in oysters was mapped after a contamination event, *i.e.* an STP overflow. NoV GII was detected to 5.29 km downstream and persisted in oysters closest to the overflow (50m) for 42 days. NoV GII concentrations decreased significantly over time: a reduction rate of 8.5% per day was observed in oysters located at two sites near the overflow ( $p < 0.001$ ) and, by comparison to published literature, suggested that GII reduction rates from oysters may be broadly similar, regardless of environmental conditions, oyster species and genotype. Five days after the overflow NoV GII concentrations decreased significantly as a function of distance at a rate of 5.8% per km ( $p < 0.001$ ), while the decline in *E. coli* concentration with distance was 20.1% per km ( $p < 0.001$ ). NoV GI was not detected. These results will help to define closure areas and durations for commercial oyster production areas following sewage overflows.

NoV contamination of oysters from Australian growing areas determined in this study seems to accord with the relatively low occurrence of 1-2 outbreaks of NoV illness annually among Australian oyster consumers. A synthesis of existing data and novel results generated in this study was undertaken (Chapter 6) to evaluate the efficacy of risk management strategies currently employed for Australian oyster harvest areas for the protection of oyster consumers from NoV, and other, infectious gastrointestinal illnesses. That analysis suggested that communication between local councils, water utility operators and shellfish authorities regarding

the reporting of sewage spills and the condition of potential sources of sewage spills (*i.e.* OSMS) is inadequate. A theoretical NoV contamination event in oyster growing areas was considered, which showed that it was possible for a small amount of NoV contaminated human faeces to cause illness among many oyster consumers. Recommendations for a risk-based virus monitoring programme were therefore proposed, including:

- i) Regulation and certification of OSMS that pose a risk of contaminating shellfish harvest areas. Closure of harvest area until certification of OSMS function is received by shellfish authorities for all OSMS that pose a risk of NoV contamination to the harvest area.
- ii) Workshops/ conferences on improving water quality for EHO and shellfish authorities
- iii) Upgrading STPs where the effluent flows into oyster growing areas
- iv) Mandatory dye studies of STP effluent flows and potential sources of sewage spills in each harvest area, to enable more effective closure times and delineation of the area closed for harvesting
- v) ASQAP to include guidance for local management committees to be formed for each growing area
- vi) ASQAP to include guidance for all oyster harvesting boats to have an on-board portable toilet/ bucket
- vii) All small marine vessels to be required to carry a portable toilet
- viii) Further studies to define the spatial and temporal distribution of NoV following raw sewage overflows
- ix) Development of an alternative indicator for NoV other than faecal coliforms *i.e.* bacteriophage testing, or for rapid, automated, remote-sensing systems to identify conditions likely to result in faecal contamination of growing areas.

## **7.2 Future directions**

In an ideal world there would be no sewage overflows or contamination of oyster harvest areas with human faeces! However they occur in Australia and with relative regularity (*see* Table 6.4). Some have impacted on oyster growing areas, resulting in contamination events in “approved” and “restricted” Australian harvest areas affecting up to many hundreds of people. The oyster



industry, local Councils and shellfish authorities cannot afford to be complacent simply because there has never been an outbreak associated with oysters from their particular harvest area. The outbreaks that have occurred, have come from undisclosed and undetected malfunctions in sewerage treatment systems and these system breakdowns can be expected to continue to occur as sewerage infrastructures age and if the functions of OSMS are not monitored.

Council EHO, and water utility operators involved with the maintenance of sewage infrastructure need to be aware of the potential consequences of sewage overflows in oyster harvest areas and ever vigilant to prevent the overflows from reaching harvest areas. Strategies for preventing sewage overflows in Australian oyster harvest areas were discussed and recommendations made in Chapter 6.

Understanding the potential contamination problems presented by STP outfalls in oyster harvest areas where STPs are not effective at removing NoV from the sewage, is essential for the safety of the Australian oyster consumer. Surveys could be conducted to determine the prevalence of NoV in oysters from Australian growing areas that have STP outfalls and the relationship of distance of the oysters from the outfall. This may help to determine whether there is a need for ongoing testing for NoV within that particular growing area, as proposed by EFSA (89).

Molecular detection methods have to date been unable to determine whether the genomic material detected, reflects the presence of infectious material. Now that NoV can be grown in cell culture (B cells) (22) it would be useful to investigate the relationship between infectious NoV (in cell culture) and genome copies detected by PCR. It may then be possible to estimate the viability of NoV in such situations as: STP effluent - to study the efficacy of inactivation of NoV in STPs; and in oysters - to determine whether NoV is inactivated before the genomic material (estimated using PCR) is eliminated.

Current NoV detection methods are not sensitive enough to detect close to the NoV ID<sub>50</sub> of 7 virus particles in contaminated oysters (108). The development of a more sensitive method may provide a better picture of contamination issues, particularly in Australia where the last two outbreaks due to NoV contaminated oysters involving hundreds of cases, had low concentrations of NoV (97, 106).

The NoV elimination estimate of 8.5% NoV per day from SRO determined in this Thesis, will enable more accurate calculation of closure times in SRO growing areas. Further studies should be conducted on other species of oysters and other genotypes of NoV under a broader range of environmental conditions to enable robust calculation of an agreed elimination rate for use

worldwide. Use of this elimination rate would help to improve prediction of elimination timeframes and the distribution in time and space of impacted areas.

The opportunity to charge a premium price for oysters grown in clean, sewage free (and NoV free) water was proposed by Dr. Doug Powell ([www.Barflog.com](http://www.Barflog.com)) at ICMSS 2013. Australian oyster growing areas have a low prevalence of NoV whereas in the UK, 38 of 39 oyster growing areas surveyed by Lowther *et al.* (176) were positive for NoV. More emphasis could be given to the low prevalence of NoV for marketing Australian oysters, but only if product safety, including development of the strategies proposed above can be assured.

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# Appendix A.1 New South Wales Oyster Sampling Plan<sup>2</sup>

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19 February 2009

## Part One – Background Information

In 1978 a multi-state outbreak of norovirus (NoV) occurred in which some 2000 people were estimated to be affected with gastroenteritis from all States of the Commonwealth, subsequent investigations determined that the oysters were harvested from the Georges river area in Sydney (285-289). Since this time food-borne virus infections have been increasingly recognised as causes of illness in humans. Between 1 January 2001 and 1 January 2008 in Australia there were 14 outbreaks of gastroenteritis related to the consumption of oysters reported to OzFoodNet; half of these outbreaks were confirmed to be due to NoV. Outbreaks of gastroenteritis are often not reported to health agencies meaning these figures likely under-estimate the true burden of enteric illness. Oysters contaminated with hepatitis A virus (HAV) have also caused outbreaks of illness in Australia; in 1997, 444 cases of HAV (including one death) were directly attributable to the consumption of oysters harvested from Wallis Lake (290).

Virus contamination of shellfish production areas is still periodically a problem in Australia; in 2008 there were two small outbreaks of NoV in New South Wales that were traced back to oysters harvested from a commercial lease in the Kalang River. Follow up PCR based testing of oysters sampled from the Kalang River detected NoV; consequently oyster farms on the Kalang River have been closed since July 2008. Oyster farms NSW1 and NSW2 were also closed previously due to the unpredictable sources of pollution.

Despite the recurrence of virus contamination in oyster production areas there is minimal information on the baseline levels of NoV or HAV in New South Wales shellfisheries. This is largely due to the expense involved in testing oysters for viruses; because there are currently no commercial laboratories in Australia that undertake NoV and HAV testing of oysters, samples are sent to New Zealand for testing.

This project involves adopting an internationally acceptable method for testing NoV and HAV in oysters at the South Australian Research and Development Institute with a view to supporting commercially oriented laboratories in Australia to implement the method in an accredited environment in out-years. The methods developed will also be used in this project to assess levels of NoV and HAV contamination in oysters in several New South Wales waterways.

### 1.1 Project Aims

- To develop and validate the 'Institute of Environmental Science and Research (ESR)' real time PCR (RT-PCR) method for NoV and HAV in oysters (291).
- To determine baseline levels of NoV and *E.coli* in oysters in the Kalang, NSW1 and NSW2 harvest areas on a routine basis during periods of increased risk of contamination.
- To determine the extent and longevity of NoV, HAV, *E.coli* and F+ RNA bacteriophage contamination of oysters following significant pollution events.
- To determine if F+ RNA bacteriophage is a useful indicator of NoV contamination following sewage spill events.

### 1.2 Project Linkages

- 'Identification of Microbial Hazards in Oysters in Australia'. Sumner and Pointon, March 2007 (SARDI)
- 'Risk based Assessment of South Australian Wild-Harvested Scallops', Madigan et al, August 2004 (SARDI)

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<sup>2</sup> Consultation on this sampling plan was undertaken with key stakeholders, including in-depth discussion at the Kalang River Working Group meeting on 5<sup>th</sup> February 2009. Subsequent changes were made to the plan to reflect the specific needs of the Group.

- 'Pilot Study of *E.coli* contamination of commercially harvested cockles'. Brake et al, August 2006 (SARDI)
- Cawthron Report No 645 'Australian Marine Biotoxin Management Plan for Shellfish Farming' 1999.

### 1.3 Project Scope

The project will include sites in three rivers in New South Wales that are not currently producing oysters for direct consumption: the Kalang River, NSW1 and NSW2.

Routine sampling will be confined to thirteen sites within these three areas:

- Seven oyster sites will be located within the Kalang River, and influent and effluent will be tested from the Urunga Sewage Treatment Plant
- Three oyster sites will be located at NSW1;
- Three oyster sites will be located at the NSW2.

Routine sampling will be done four times with monthly intervals starting in December 2009 at the selected sites in the harvest areas. Routine sampling in the Kalang River and Urunga Sewage Treatment Plant will be started in January 2009 and will be undertaken five times on occasions targeted to 'medium risk' of pollution e.g. increased numbers of people staying in the catchment, higher levels of rainfall etc.

Sampling in New South Wales will also be carried out after two suspected adverse pollution events of 'significant impact' (defined later).

Oyster flesh obtained during the routine sampling shall be analysed for NoV and *E.coli*. The oyster flesh obtained during adverse pollution events will be analysed for NoV, *E.coli*, HAV and F<sup>+</sup> RNA bacteriophage.

Results will be analysed and reported to the Project Team.

Assessing virus content of water samples is outside the scope and resources available in this study.

### 1.4 Project Timeframe

- a) Kalang River. Routine sampling will begin in January 2009 and continue until five sampling events have occurred (no later than January 2010). Reporting of the results to the Project Team will be no later than three weeks after sample receipt, and brief reports identifying progress and issues will be given to the Project Team routinely on a two monthly basis.
- b) NSW1 and NSW1 harvest areas. Routine sampling will begin in December 2009 and continue until April 2010. This period has been identified as a time when higher than usual levels of rainfall occur, and thus may represent a higher level of risk of virus contamination of oysters. Reporting of the results will be no later than two months after analysis of the last sample received.

### 1.5 Project Team

Name	Expected Contribution
NSW Farmers Association and Kalang River Oyster Farmers,.	In conjunction with NSWFA arrange collection of oyster samples from the Kalang, NSW1 and NSW2 harvest areas and send to SARDI
University of Tasmania and (Felicity Brake – PhD Candidate, Tom Ross – PhD	Analyse targeted oyster samples and suspected pollution event samples for viral



Supervisor)	and bacterial contamination.
SARDI (Catherine McLeod – PhD Supervisor)	Facilitate the project implementation and reporting.
NSW Food Authority (Anthony Zammit and Phil Baker)	Determine sampling sites, and frequencies in conjunction with SARDI and facilitate sampling. Provision of hydrographic and meteorological data.
Kalang River Working Group (John Williams)	<p>Input into sample site selection, and provision of hydrographic and meteorological data e.g. temperature, salinity, wind, tide, cloud cover, rainfall etc.</p> <p>Facilitate taking samples of effluent and influent from the Urunga Sewage Treatment Plant.</p> <p>Facilitate monitoring of anti diarrhoeal sales from pharmacies in Urunga<sup>3</sup>.</p>

## Part Two - Project Implementation Plan

### 2.1 Routine Sampling Plan

#### **a) Site Determination and Location**

The location of each sample site in the Kalang, NSW1 and NSW 2 harvest areas has been discussed and sites will be selected using the collective knowledge and information available to the project team.

Information considered was based on historical monitoring data collected between the project team agencies identifying areas of shellfish contamination and environmental contamination from known or unknown sources; location of commercially active oyster leases; potential contamination sources; freshwater influences; tidal influences; quantity of oysters to sustain the sampling regime and the distribution of sites between reticulated and septic tank communities.

Routine sampling of oysters will be confined to thirteen sites within the three rivers. Seven sites will be located within the Kalang River, three sites at NSW1 and three sites at the NSW2. The rivers to be sampled are identified on the appended maps<sup>4</sup> (Appendices 2-4).

A control site was not included because all sites would potentially be affected by runoff contamination.

#### **b) Frequency**

The extent of the oyster sampling was considered in consultation with SARDI statisticians.

Five samples of oysters will be collected from each of the 13 sites on each sampling occasion. Three samples of influent (raw sewage) and three samples of effluent (from the discharge pipe) will be collected from the Urunga Sewage Treatment Plant on each of four sampling occasions.

Routine sampling for the **Kalang River** will start in January 2009 and will occur on five separate occasions during times of 'medium pollution risk' as ascertained by the project team over a 12-month

<sup>3</sup> Monitoring anti-diarrhoeal sales from local pharmacies requires further discussion to fully assess feasibility.

<sup>4</sup> Specific sites were discussed during field visits in February 2009. Further analysis is required to determine exact location of the sites within the river systems.

period. 'Medium pollution risk'<sup>5</sup> will include times such as: (a) when the population in the catchment has increased (such as summer holiday periods), or (b) when rainfall is elevated slightly above normal levels.

Routine sampling in the NSW1 and NSW2 harvest areas will occur on four occasions at monthly intervals in December 2009 and January, February and March 2010.

### **c) Responsibility for Sampling and Analysis**

NSW Farmers Association and Kalang River Oyster Farmers in conjunction with the NSW Food Authority will arrange for the collection and transportation of the samples to SARDI in Adelaide.

Oysters will be collected from the Kalang River in January 2009 and stored frozen at minus 20 °C. These will be transported as per the SARDI Protocol for Shellfish Collection (Appendix 1). All other oyster samples will be collected "live" and transported as per the SARDI Protocol for Shellfish Collection (Appendix 1).

Oyster flesh obtained during the routine sampling shall be analysed for NoV and *E.coli*. Oysters taken during the final sampling round (Kalang River only) will also be tested for additional viruses associated with sewage contamination.

The extraction of NoV from oysters will be carried out using a protease digestion method described by Greening and Hewitt (2008) (291). Real time PCR amplification of NoV genogroup I and II strains will also be carried out as per Greening and Hewitt (2008).

*E. coli* analysis of oysters will be undertaken using the method described in ISO/TS 16649-3:2005 (E) and Donovan et al (1998) (292). The sampling and analysis results will be collated and interpreted by SARDI.

## **2.2 Adverse Pollution Event Sampling Plan**

Comprehensive sampling will be undertaken following adverse pollution events. The number of events monitored, as part of the project is likely to be restricted to a total number of 2 separate events due to resource limitations. Adverse pollution events likely to be the most significant will be targeted. Such as, the first significant rainfall after a dry spell or a sewage overflow which is likely to have implications on a wide environmental area.

The most likely adverse pollution source in the NSW1 and NSW2 areas is a spill of raw sewage through a sewage treatment plant (STP) outfall into the marine environment. Accordingly, an 'Adverse Pollution Event'<sup>6</sup> for the purpose of this project is defined as:

A sewage overflow from > 50 cubic metres in volume; or

A large rainfall event in which significant sewage contamination has occurred (e.g. greater than 100 ml in 24 hrs).

Discussion between the Project Team will determine whether an event is sufficiently significant for the purposes of inclusion in the project.

### **(a) Site Determination and location.**

The number and location of sampling sites selected is intended to be adequate to produce the data necessary to effectively evaluate the effect of point sources of pollution on oysters. A

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<sup>5</sup> 'Medium pollution risk' times are distinct from 'Adverse Pollution Events'; 'Adverse Pollution Events' are much larger scale contamination events (defined in next section).

<sup>6</sup> The definition of an Adverse Pollution Event was discussed and agreed with the Project Team

gradient approach through space and time will be taken in response to an adverse pollution event.

Five sites within the oyster beds affected by the pollution event should be sampled. These sites should be moving away from the source of pollution at regular distance intervals, if oyster availability permits. Single samples (each comprising a minimum of 22 large oysters, 30 medium oysters, or 60 small oysters) will be taken at each of the five sites on 8 occasions.

#### (b) Frequency

The extent of the oyster sampling was considered in consultation with SARDI statisticians.

Once an adverse pollution event has been identified for inclusion, sampling will be undertaken as soon as the event ceases but within 24 hours of the event and again at ~ 48 hours, 72 hours, 7 days, 2 weeks, 4 weeks and at 2 and 3 months for a total of 8 occasions. Ideally the first sample should be taken as soon as possible to reduce dilution influences by dispersion. Little information is known about the dispersion and dilution of viruses in seawater and oysters, therefore sampling times may be adjusted after the first Adverse Pollution Event. If two consecutive samples taken from the same site are negative, sampling will cease.

#### (c) Responsibilities for Sampling and Analysis

NSW Farmers Association and Kalang River Oyster Farmers in conjunction with the NSW Food Authority will arrange for sample collection and transportation to SARDI in Adelaide as per the SARDI Protocol for Shellfish Collection (Appendix One).

The location of each sample site should be clearly noted, the distance of the sampling site from the source of pollution (e.g. broken pipe) noted, and a map showing the location of the sample sites and pollution source drawn.

The oyster flesh obtained during adverse pollution events will be analysed for NoV, *E.coli*, HAV and F<sup>+</sup> RNA bacteriophage.

The extraction of NoV and HAV from oysters will be carried out using a protease digestion method described by Greening and Hewitt (2008) (291). Real time PCR amplification of NoV genogroup I and II strains will also be carried out as per Greening and Hewitt (2008). PCR amplification of HAV will be carried out using primers that are utilised for routine clinical sample testing at the Institute of Medical and Veterinary Science (Adelaide).

*E. coli* analysis of oysters will be undertaken using the method described in ISO/TS 16649-3:2005 (E) and Donovan et al (1998) (292).

Presence of F<sup>+</sup> RNA coliphage will be determined using an ISO standard method for bacteriophage detection in shellfish.

The sampling and analysis results will be collated and interpreted by SARDI.

## 2.3 Results and Feedback

**Kalang River:** SARDI will report the results of the routine sampling to the Project Team no later than three weeks after sample receipt, and brief reports identifying progress and issues will be given to the Project Team on a two monthly basis.

**NSW1 and NSW2 harvest areas:** SARDI will provide the Project Team with an interim progress report within two months of completing the routine sampling programme.

On completion of the overall project, a report detailing project outcomes and recommendations will be provided to the Project Team.

## 2.4 Information and Sample Collection

a) The NSW Food Authority and KRWG will provide:

Hydrographic and meteorological data throughout the project to assist the identification of environmental conditions that affect the contamination of oysters. This will include details of the following parameters at sampling times:

General weather conditions e.g. rain, cloud cover etc

Air temperature

Seawater temperature

Salinity of seawater

Turbidity of water at sampling site (visual estimate)

State of tide e.g. incoming etc

Rainfall in the preceding 24 - 48 hours

- It is important to note that while many of these estimates appear to be subjective it is surprising how these observations can lead to good associations with contamination if they are consistently and systematically reported and recorded.

b) The NSW Food Authority, NSW Farmers Association and Kalang River Oyster Farmers, will facilitate the sampling of oysters at stipulated times and transportation of samples to SARDI.

c) The KRWG will facilitate (a) the collection of information on anti diarrhoeal sales from pharmacies in Urunga township; (b) information about disease outbreaks of NoV within the community in the Kalang river catchment; and (c) samples to be taken from the Urunga Sewage Treatment Plant. This will assist in identifying whether there is any association between the presence of viruses in oysters and enteric illness in the community.

## Appendix One: SARDI Protocol for Shellfish Collection

### a) Collection of routine samples

Collect 5 samples of oysters per collection site.

Collect a minimum of 16 large oysters, 25 medium oysters or 50 small oysters for each sample. This amount will allow each sample to be analysed for NoV and *E.coli*.

Collect 3 samples comprising 40 mL sewage influent and 3 samples comprising 40 mL sewage effluent.

### b) Collection of adverse pollution event samples

Collect a minimum of 22 large oysters, 30 medium oysters, or 60 small oysters for each sample. This amount will allow each sample to be analysed for NoV, HAV, *E.coli*, and F-RNA bacteriophage.

The location of each sample site should be clearly noted, the distance of the sampling site from the source of pollution (e.g. broken pipe) noted, and a map showing the location of the sample sites and pollution source drawn.

### c) Sample Packing and Labeling

Pack each sample into separately labelled leak-proof plastic bags - do not mix sites.

For each sample complete the label on the front of the bag with the date and time of collection, and sample site. The location of sampling sites should be kept the same throughout the study. If oysters are depleted at a sampling site, they should be taken from the nearest possible alternative site.

Shellfish samples are to be transported by overnight courier to SARDI in leak-proof esky's with freezer pads. Samples should go by courier on the same day they are collected.

SARDI staff must be notified if there are delays in shipment.

### d) Address for Shipping

Attention: Felicity Brake/ Dr. Cath McLeod  
SARDI Food Innovation and Safety  
33 Flemington Street  
Glenside SA

#### Contact details

Felicity Brake 08-82077838 mobile 0448 890 273  
Dr. Cath McLeod 08-82077904 mobile 0429 814 217  
Food Safety Fax:08-82077854

### e) Additional notes

If samples are collected before a weekend or during holidays, they may be stored at  $5 \pm 3^{\circ}\text{C}$  for up to 48 hours. Please discuss this with Felicity Brake or Cath McLeod before collection. Samples showing evidence of inappropriate storage either prior to or during shipment to the laboratory may be rejected. Samples that are in any state of decomposition (by sight or smell) may be rejected.

Insufficient sample: under most circumstances, 16 large shellfish is the minimum quantity required for acceptance for routine testing. Only shellfish digestive tissue is used for viral testing and a minimum of 5 grams of digestive tissue and 6 oysters is required. A minimum of 200 g and 10 whole oysters is required for *E. coli* testing.

Samples may be rejected if:

- No request for or insufficient details are received
- The shellfish sample does not match the accompanying request form (wrong description of sample)

- Shellfish sample is not labelled
- Shellfish labelling is illegible
- Identification on the shellfish sample is not unique
- Less quantity than requested maybe processed following discussion with the client

# Appendix A.2 Oyster Sampling Plan for South Australia

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January 2010

## 1. Part One – Background Information

In 1978 a multi-state outbreak of norovirus (NoV) occurred in which some 2000 people were estimated to be affected with gastroenteritis from all States of the Commonwealth, subsequent investigations determined that the oysters were harvested from the Georges river area in Sydney, (Murphy 1978, Linco & Grohmann 1980). Since this time food-borne virus infections have been increasingly recognised as causes of illness in humans. Between 1 January 2001 and 1 January 2008 in Australia there were 15 outbreaks of gastroenteritis related to the consumption of oysters reported to OzFoodNet; half of these outbreaks were confirmed to be due to NoV. Outbreaks of gastroenteritis are often not reported to health agencies meaning that these figures are likely to be an under-estimate of the true burden of enteric illness. Oysters contaminated with hepatitis A virus (HAV) have also caused outbreaks of illness in Australia; in 1997, 444 cases of HAV (including one death) were directly attributable to the consumption of oysters harvested from Wallis Lake, (Conaty 2000).

Virus contamination of shellfish production areas is still periodically a problem in Australia; in 2007 there was an outbreak of NoV associated with the consumption of oysters from NSW and a further two small outbreaks of NoV in New South Wales that were traced back to oysters harvested from a commercial lease in the Kalang River, (Huppertz *et al.* 2008, NSWFA 2008).

There is minimal information on the baseline levels of NoV or HAV in Australian shellfisheries. This is largely due to the expense involved in testing oysters for viruses, as there are currently no commercial laboratories in Australia that perform the tests. Samples have in the past been sent to New Zealand for testing.

No virus contamination has been traced to oyster farms in South Australia. However, oyster leases have been closed for harvesting, due to high *E. coli* counts. Virus testing of the shellfish has not previously been conducted in South Australia.

This project has involved adopting an internationally acceptable method for testing NoV in oysters at the South Australian Research and Development Institute (SARDI) with a view to supporting commercially oriented laboratories in Australia to implement the method. The method has been introduced at SARDI and will be used in this project to assess levels of NoV contamination in oysters in key South Australian shellfish growing areas.

### 1.1 Aim

To determine baseline levels of NoV and *E. coli* in oysters from South Australian oyster shellfisheries on a routine basis during periods of increased risk of contamination.

## 1.2 Project Linkages

‘Identification of Microbial Hazards in Oysters in Australia’. Sumner and Pointon, March 2007 (SARDI)

‘Risk based Assessment of South Australian Wild-Harvested Scallops’, Madigan et al, August 2004 (SARDI)

‘Pilot Study of *E. coli* contamination of commercially harvested cockles’. Brake *et al.* August 2006 (SARDI)

Cawthron Report No 645 ‘Australian Marine Biotoxin Management Plan for Shellfish Farming’ 1999.

## 1.3 Project Scope

The project will sample from two oyster shellfisheries in South Australia.

Routine sampling will be confined to five samples within these two areas:

One oyster site will be in SA1 Harvesting Area.

One oyster site will be in the SA2 Harvesting Area.

Routine sampling in the South Australian shellfisheries will be started in 2010 and will be undertaken four times on occasions targeted to ‘medium risk’ of pollution e.g. increased numbers of people staying in the catchment, higher levels of rainfall etc.

Oyster flesh obtained during the routine sampling shall be analysed for NoV and *E. coli*.

Results will be analysed and reported to the Program Leader of the South Australian Shellfish Quality Assurance Program at the end of the project.

## 1.4 Project Timeframe

Routine sampling will begin in January 2010 and continue until four sampling events have occurred (no later than January 2011). Reporting of the results to the Project Team will occur at the end of the project.

## 1.5 Project Team

<b>Name</b>	<b>Expected Contribution</b>
PIRSA – Clinton Wilkinson	In conjunction with Glen Boucher arrange collection of oyster samples from the SA oyster shellfisheries and send to SARDI
University of Tasmania and (Felicity Brake – PhD Candidate, Tom Ross –	Analyse targeted oyster samples and suspected pollution event samples for viral and bacterial contamination.



PhD Supervisor)	Facilitate the project implementation and reporting.
SARDI (Catherine McLeod – PhD Supervisor)	Facilitate the project implementation and reporting.
PIRSA - Clinton Wilkinson	Determine sampling sites, and frequencies in conjunction with SARDI and facilitate sampling. Provision of hydrographic and meteorological data. Input into sample site selection and provision of hydrographic and meteorological data e.g. temperature, salinity, wind, tide, cloud cover, rainfall etc. Facilitate taking water samples from Miniribbie Creek.

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## Part Two - Project Implementation Plan

### 2.1 Routine Sampling Plan

#### a) Site Determination and Location

The location of each sample site in South Australia has been selected using the collective knowledge and information available to the project team.

Information considered was based on historical monitoring data collected between the project team agencies identifying areas of shellfish contamination and environmental contamination from known or unknown sources; potential contamination sources; freshwater influences; tidal influences; quantity of oysters to sustain the sampling regime and the distribution of sites between reticulated and septic tank communities.

Routine sampling of oysters will be confined to two sites within South Australia. One site will be located within SA1 Harvesting Area and the other site will be in the SA2 Harvesting Area.

#### b) Frequency

The extent of the oyster sampling was considered in consultation with SARDI statisticians.

Five samples of oysters will be collected from the two sites on each of four sampling occasions.

Routine sampling in South Australia will start in January 2010 and will occur on four separate occasions during times of 'medium pollution risk' as set out in the table below. 'Medium pollution risk' will include times such as: (a) when the population in the catchment has increased (such as summer holiday periods), or (b) when rainfall is elevated slightly above normal levels.

### Sample Collection Dates

Area to be sampled	Summer sampling	Autumn/easter sampling	Winter sampling	Spring sampling
SA1	1 <sup>st</sup> February	3 <sup>rd</sup> May	2 <sup>nd</sup> August	20 <sup>th</sup> September
SA2	1 <sup>st</sup> February	3 <sup>rd</sup> May	2 <sup>nd</sup> August	20 <sup>th</sup> September

### c) Responsibility for Sampling and Analysis

Clinton Wilkinson PIRSA in conjunction with Glen Boucher will arrange for the collection and transportation of the samples to SARDI in Adelaide.

Oyster samples will be collected “live” and transported as per the SARDI Protocol for Shellfish Collection (Appendix 1).

Oyster flesh obtained during the routine sampling shall be analysed for NoV and *E.coli* at SARDI Food Safety. The extraction of NoV from oysters will be carried out using a protease digestion method followed by real time PCR amplification of NoV genogroup I and II strains as described in Greening and Hewitt (2008).

*E. coli* analysis of oysters will be undertaken using the method described in ISO/TS 16649-3:2005 and Donovan *et al* (1998).

The sampling and analysis of results will be collated and interpreted by SARDI.

## 2.3 Results and Feedback

Site 1 & 2:

Positive results will be confirmed by repeating the reverse transcription and Real-Time PCR on the RNA isolated from the oyster digest. If a repeat test is positive then the sample will be reported as positive.

Results will be analysed and reported to the Program Leader of the South Australian Shellfish Quality Assurance Program (SASQAP), Clinton Wilkinson at the end of the project. The report detailing project outcomes and recommendations will be provided to the Program Leader of SASQAP, Clinton Wilkinson.

## 2.4 Information and Sample Collection

a) Clinton Wilkinson PIRSA will provide\*:

Hydrographic and meteorological data throughout the project to assist the identification of environmental conditions that affect the contamination of oysters. This will include details of the following parameters at sampling times:

General weather conditions e.g. rain, cloud cover etc

Air temperature

Seawater temperature

Salinity of seawater

Turbidity of water at sampling site (visual estimate)

State of tide e.g. incoming etc

Rainfall in the preceding 24 - 48 hours

It is important to note that while many of these estimates appear to be subjective it is surprising how these observations can lead to good associations with contamination if they are consistently and systematically reported and recorded.

b) Clinton Wilkinson PIRSA will facilitate the sampling of oysters at stipulated times and transportation of samples to SARDI.

c) Clinton Wilkinson PIRSA will facilitate sampling from any suspected pollution sources. This will assist in identifying whether there is any association between the presence of viruses in oysters and enteric illness in the community.

\* The feasibility of obtaining hydrographic and meteorological data is to be discussed with Clinton Wilkinson

## Appendix One: SARDI Protocol for Shellfish Collection

a) Collection of routine samples

Collect 5 samples of oysters per collection site on the dates specified in the table set out below.

Collect a minimum of 16 large oysters, 25 medium oysters or 50 small oysters for each sample. This amount will allow each sample to be analysed for NoV and *E.coli*.

Sample Collection Dates

Area to be sampled	Summer sampling	Autumn/easter sampling	Winter sampling	Spring sampling
SA1	1 <sup>st</sup> February	3 <sup>rd</sup> May	2 <sup>nd</sup> August	20 <sup>th</sup> September
SA2	1 <sup>st</sup> February	3 <sup>rd</sup> May	2 <sup>nd</sup> August	20 <sup>th</sup> September

b) Sample Packing and Labeling

Pack each sample into separately labelled leak-proof plastic bags - do not mix sites.

For each sample complete the label on the front of the bag with the date and time of collection, and sample site. The location of sampling sites should be kept the same throughout the study. If oysters are depleted at a sampling site, they should be taken from the nearest possible alternative site.

Shellfish samples are to be transported by overnight courier to SARDI in leak-proof esky's with freezer pads. Samples should go by courier on the same day they are collected.

SARDI staff must be notified if there are delays in shipment.

#### c) Address for Shipping

Attention: Felicity Brake/ Dr. Cath McLeod  
SARDI Food Innovation and Safety  
33 Flemington Street  
Glenside SA

#### Contact details

Felicity Brake 08-82077838 mobile work 0439416216 w/ends etc 0448890273  
Dr. Cath McLeod 08-82077904 mobile 0429814217

#### d) Additional notes

If samples are collected before a weekend or during holidays, they may be stored at  $5 \pm 3$  °C for up to 48 hours. Please discuss this with Felicity Brake or Cath McLeod before collection. Samples showing evidence of inappropriate storage either prior to or during shipment to the laboratory may be rejected. Samples that are in any state of decomposition (by sight or smell) may be rejected.

Insufficient sample: under most circumstances, 16 large shellfish is the minimum quantity required for acceptance for routine testing. Only shellfish digestive tissue is used for viral testing and a minimum of 5 grams of digestive tissue and 6 oysters is required. A minimum of 200 g and 10 whole oysters is required for *E. coli* testing.

Samples may be rejected if:

No request for or insufficient details are received

The shellfish sample does not match the accompanying request form

Shellfish sample is not labelled

Shellfish labelling is illegible

Identification on the shellfish sample is not unique

Less quantity than requested maybe processed following discussion with the client.

# Appendix A.3 Oyster Sampling Plan for Tasmania

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**January 2010**

## Part One – Background Information

In 1978 a multi-state outbreak of norovirus (NoV) occurred in which some 2000 people were estimated to be affected with gastroenteritis from all States of the Commonwealth, subsequent investigations determined that the oysters were harvested from the Georges river area in Sydney, (Murphy 1978, Linco & Grohmann 1980). Since this time food-borne virus infections have been increasingly recognised as causes of illness in humans. Between 1 January 2001 and 1 January 2009 in Australia there were 15 outbreaks of gastroenteritis related to the consumption of oysters reported to OzFoodNet, half of these outbreaks were confirmed to be due to NoV. Outbreaks of gastroenteritis are often not reported to health agencies meaning these figures likely underestimate the true burden of enteric illness.

Virus contamination of shellfish production areas is still periodically a problem in Australia; in 2007 there was an outbreak of norovirus associated with the consumption of oysters from NSW and a further two small outbreaks of NoV in New South Wales that were traced back to oysters harvested from a commercial lease in the Kalang River, (Huppatz *et al.* 2008, NSWFA 2008).

There is minimal information on the baseline levels of NoV in Tasmanian shellfisheries. This is largely due to the expense involved in testing oysters for viruses. Up until now, there have been no commercial laboratories in Australia that perform the tests. Samples have been sent to New Zealand for testing.

No virus contamination has been traced to oyster farms in Tasmania, however oyster leases have been closed previously, due to pollution from a variety of sources. Virus testing of shellfish has not previously been conducted in Tasmania.

This project has involved adopting an internationally acceptable method for testing NoV in oysters at the South Australian Research and Development Institute (SARDI) with a view to supporting commercially oriented laboratories in Australia to implement the method. The method has been introduced at SARDI and will be used in this project to assess the levels of NoV contamination in oysters in key Tasmanian shellfish growing areas.

### 1.1 Aim

To determine baseline levels of NoV and *E.coli* in oysters from two key Tasmanian oyster shellfisheries on a routine basis during periods of increased risk of contamination.

## 1.2 Project Linkages

‘Identification of Microbial Hazards in Oysters in Australia’. Sumner and Pointon, March 2007 (SARDI)

‘Risk based Assessment of South Australian Wild-Harvested Scallops’, Madigan et al, August 2004 (SARDI)

‘Pilot Study of *E.coli* contamination of commercially harvested cockles’. Brake et al, August 2006 (SARDI)

Cawthron Report No 645 ‘Australian Marine Biotxin Management Plan for Shellfish Farming’ 1999.

## 1.3 Project Scope

Samples will be taken from two oyster production areas in Tasmania.

Routine sampling will be confined to five samples from each of two sites:

One site will be at TAS1

One site will be located at TAS2

Routine sampling in the Tasmanian shellfisheries will be started in 2010 and will be undertaken four times on occasions targeted to ‘medium risk’ of pollution e.g. increased numbers of people staying in the catchment, higher levels of rainfall etc.

Oyster flesh obtained during the routine sampling shall be analysed for NoV and *E.coli*.

Results will be analysed and reported to the Tasmanian Oyster Shellfisheries Manager, Ray Brown at the end of the project.

## 1.4 Project Timeframe

Routine sampling will begin in February 2010 and continue until four sampling events have occurred (no later than January 2011). A brief report identifying results and issues will be given to the Project Team at the end of the testing period.

## 1.5 Project Team

Name	Expected Contribution
University of Tasmania and (Felicity Brake – PhD Candidate, Tom Ross – PhD Supervisor)	Analyse targeted oyster samples and suspected pollution event samples for viral and bacterial contamination. Facilitate the project implementation and reporting.

SARDI (Catherine McLeod – PhD Supervisor)	Facilitate the project implementation and reporting.
TAS DHHS-Ray Brown	Determine sampling sites and frequencies in conjunction with SARDI. Facilitate sampling and forward samples to SARDI. Provision of hydrographic and meteorological data. Facilitate sampling of effluent and influent from the Sewage Treatment Plant.
TAS Oyster farmers	Provision of samples.

## Part Two - Project Implementation Plan

### 2.1 Routine Sampling Plan

#### a) Site Determination and Location

The location of each sample site in Tasmania has been selected using the collective knowledge and information available to the project team.

Information considered was based on historical monitoring data collected between the project team agencies identifying areas of shellfish contamination and environmental contamination from known or unknown sources; potential contamination sources; freshwater influences; tidal influences; quantity of oysters to sustain the sampling regime and the distribution of sites between reticulated and septic tank communities.

Routine sampling of oysters will be confined to two sites within Tasmania. One site will be located at TAS1 and the other site will be at TAS2.

#### b) Frequency

The extent of the oyster sampling was considered in consultation with SARDI statisticians.

Five samples of oysters will be collected from the two sites on each of four sampling occasions. Three samples of influent (raw sewage) and three samples of effluent (from the discharge pipe) will be collected from the associated Sewage Treatment Plant or suspected pollution source on each of four sampling occasions.

Routine sampling in Tasmania will start in February 2010 and will occur on four separate occasions during times of 'medium pollution risk' as set out in the table below. 'Medium pollution risk' will include times such as: (a) when the population in the catchment has increased (such as summer holiday periods), or (b) when rainfall is elevated slightly above normal levels.

#### Sample Collection Dates

Area to be sampled	Summer sampling	Autumn/easter sampling	Winter sampling	Spring sampling
TAS1	8 <sup>th</sup> February	26 <sup>th</sup> April	26 <sup>th</sup> July	13 <sup>th</sup> September
TAS2	8 <sup>th</sup> February	26 <sup>th</sup> April	26 <sup>th</sup> July	13 <sup>th</sup> September

### c) Responsibility for Sampling and Analysis

The DHHS in conjunction with the TAS Farmers Association will arrange for the collection and transportation of the samples to SARDI in Adelaide.

Oyster samples will be collected “live” and transported as per the SARDI Protocol for Shellfish Collection (Appendix 1).

Oyster flesh obtained during the routine sampling shall be analysed for NoV and *E. coli*. The extraction of NoV from oysters will be carried out using a protease digestion method followed by real time PCR amplification of NoV genogroup I and II strains as described in Greening and Hewitt (2008).

*E. coli* analysis of oysters will be undertaken using the method described in ISO/TS 16649-3:2005 and Donovan *et al.* (1998).

Sewage samples will be stored frozen at minus 20<sup>0</sup>C at SARDI until they are tested for NoV. It is anticipated that a method for testing sewage for NoV will be introduced at SARDI as part of the PhD project.

The sampling and analysis results will be collated and interpreted by SARDI

## 2.2 Results and Feedback

### Site 1 & 2:

Positive results will be confirmed by repeating the reverse transcription and Real-Time PCR on the RNA isolated from the oyster digest. If a repeat test is positive then the sample will be reported as positive.

On completion of the overall project, a report detailing project outcomes and recommendations will be provided to the DHHS - Ray Brown.

## 2.3 Information and Sample Collection

a) The TAS Farmers Association and the DHHS will provide<sup>7</sup>:

Hydrographic and meteorological data throughout the project to assist the identification of environmental conditions that affect the contamination of oysters. This will include details of the following parameters at sampling times:

General weather conditions e.g. rain, cloud cover, temperature etc

Seawater temperature

Salinity of seawater

Turbidity of water at sampling site (visual estimate)

State of tide e.g. incoming etc

Rainfall in the preceding 24 - 48 hours

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<sup>7</sup> The feasibility of obtaining hydrographic and meteorological data is to be discussed with Ray Brown.



It is important to note that while many of these estimates appear to be subjective it is surprising how these observations can lead to good associations with contamination if they are consistently and systematically reported and recorded.

b) The Tas DHHS and TAS Oyster farmers will facilitate the sampling of oysters at stipulated times and transportation of samples to SARDI. Sampling dates are listed in the table in Part 2.1 (b)

c) The Tasmanian DHHS will facilitate sampling from any associated Sewage Treatment Plants or suspected pollution sources. This will assist in identifying whether there is any association between the presence of viruses in oysters and enteric illness in the community.

## Appendix One: SARDI Protocol for Shellfish Collection

### a) Collection of routine samples

Collect 5 samples of oysters per collection site (i.e. 5 x 16 large oysters per site) on the dates set out in the table below.

Collect a minimum of 16 large oysters, 25 medium oysters or 50 small oysters for each sample. This amount will allow each sample to be analysed for NoV and *E.coli*.

Collect 3 samples comprising 1L sewage influent and 3 samples comprising 1L sewage effluent.

### Sample Collection Dates

Area to be sampled	Summer sampling	Autumn/easter sampling	Winter sampling	Spring sampling
TAS1	8 <sup>th</sup> February	26 <sup>th</sup> April	26 <sup>th</sup> July	13 <sup>th</sup> September
TAS2	8 <sup>th</sup> February	26 <sup>th</sup> April	26 <sup>th</sup> July	13 <sup>th</sup> September

### b) Sample Packing and Labeling

Pack each sample into separately labelled leak-proof plastic bags - do not mix sites.

For each sample complete the label on the front of the bag with the date and time of collection, and sample site. The location of sampling sites should be kept the same throughout the study. If oysters are depleted at a sampling site, they should be taken from the nearest possible alternative site.

Shellfish samples are to be transported by overnight courier to SARDI in leak-proof esky's with freezer pads. Samples should go by courier on the same day they are collected.

SARDI staff must be notified if there are delays in shipment.

c) Address for Shipping

Attention: Felicity Brake/ Dr. Cath McLeod  
SARDI Food Innovation and Safety  
33 Flemington Street  
Glenside SA  
Contact details  
Felicity Brake 08-82077838 mobile 0448 890 273  
Dr. Cath McLeod 08-82077904 mobile 0429 814 217

d) Additional notes

If samples are collected before a weekend or during holidays, they may be stored at  $5 \pm 3^{\circ}\text{C}$  for up to 48 hours. Please discuss this with Felicity Brake or Cath McLeod before collection. Samples showing evidence of inappropriate storage either prior to or during shipment to the laboratory may be rejected. Samples that are in any state of decomposition (by sight or smell) may be rejected.

Insufficient sample: under most circumstances, 16 large shellfish is the minimum quantity required for acceptance for routine testing. Only shellfish digestive tissue is used for viral testing and a minimum of 5 grams of digestive tissue and 6 oysters is required. A minimum of 200 g and 10 whole oysters is required for *E. coli* testing.

Samples may be rejected if:

No request for or insufficient details are received

The shellfish sample does not match the accompanying request form

Shellfish sample is not labelled

Shellfish labelling is illegible

Identification on the shellfish sample is not unique

Less quantity than requested maybe processed following discussion with the client.

# Appendix A:4 Oyster testing for Chapter 3. Results of *E. coli* and NoV GI & GII Testing

Laboratory Number	Sample Type	Kalang Sample Type	Kalang Sample Site	Collected	<i>E.coli</i> MPN/100g	NoVGI	NoV GII
FS09-0153	oysters	Kalang routine 1	K4-1	5/02/2009	<20	< RLOD	< RLOD
FS09-0154	oysters	Kalang routine 1	K4-2	5/02/2009	20	< RLOD	<RLOD
FS09-0150	oysters	Kalang routine 1	K4-3	5/02/2009	<20	< RLOD	<RLOD
FS09-0152	oysters	Kalang routine 1	K4-4	5/02/2009	<20	< RLOD	< RLOD
FS09-0155	oysters	Kalang routine 1	K4-5	5/02/2009	<20	< RLOD	< RLOD
FS09-0156	oysters	Kalang routine 1	K5-1	5/02/2009	70	< RLOD	< RLOD
FS09-0157	oysters	Kalang routine 1	K5-2	5/02/2009	<20	< RLOD	< RLOD
FS09-0158	oysters	Kalang routine 1	K5-3	5/02/2009	110	< RLOD	< RLOD
FS09-0159	oysters	Kalang routine 1	K5-4	5/02/2009	20	< RLOD	< RLOD
FS09-0160	oysters	Kalang routine 1	K5-5	5/02/2009	20	< RLOD	< RLOD
FS09-0161	oysters	Kalang routine 1	K7-1	5/02/2009	<20	< RLOD	< RLOD
FS09-0162	oysters	Kalang routine 1	K7-2	5/02/2009	20	< RLOD	< RLOD
FS09-0163	oysters	Kalang routine 1	K7-3	5/02/2009	<20	< RLOD	< RLOD
FS09-0164	oysters	Kalang routine 1	K7-4	5/02/2009	40	< RLOD	< RLOD
FS09-0165	oysters	Kalang routine 1	K7-5	5/02/2009	<20	< RLOD	< RLOD
FS09-0178	oysters	Kalang routine 1	K3-1	5/02/2009	<20	<RLOD	<RLOD
FS09-0179	oysters	Kalang routine 1	K3-2	5/02/2009	20	<RLOD	< RLOD
FS09-0180	oysters	Kalang routine 1	K3-3	5/02/2009	20	<RLOD	< RLOD
FS09-0181	oysters	Kalang routine 1	K3-4	5/02/2009	50	<RLOD	< RLOD
FS09-0151	oysters	Kalang routine 1	K3-5	5/02/2009	<20	<RLOD	< RLOD
FS09-0166	oysters	Kalang routine 1	K6-3	5/02/2009	40	<RLOD	< RLOD
FS09-0167	oysters	Kalang routine 1	K6-4	5/02/2009	40	<RLOD	< RLOD
FS09-0168	oysters	Kalang routine 1	K2-1	5/02/2009	110	<RLOD	< RLOD
FS09-0169	oysters	Kalang routine 1	K2-2	5/02/2009	<20	<RLOD	< RLOD
FS09-0170	oysters	Kalang routine 1	K2-3	5/02/2009	40	<RLOD	< RLOD
FS09-0171	oysters	Kalang routine 1	K2-4	5/02/2009	40	<RLOD	< RLOD
FS09-0172	oysters	Kalang routine 1	K2-5	5/02/2009	70	<RLOD	< RLOD
FS09-0173	oysters	Kalang routine 1	K8-1	5/02/2009	<20	<RLOD	< RLOD
FS09-0174	oysters	Kalang routine 1	K8-2	5/02/2009	<20	<RLOD	< RLOD
FS09-0175	oysters	Kalang routine 1	K8-3	5/02/2009	<20	<RLOD	< RLOD
FS09-0176	oysters	Kalang routine 1	K8-4	5/02/2009	<20	<RLOD	< RLOD
FS09-0177	oysters	Kalang routine 1	K8-5	5/02/2009	<20	<RLOD	< RLOD

FS09-0182	oysters	Kalang Adverse 1	K4-1	2/03/2009	5400	<RLOD	< RLOD
FS09-0183	oysters	Kalang Adverse 1	K4-2	2/03/2009	3500	<RLOD	< RLOD
FS09-0184	oysters	Kalang Adverse 1	K4-3	2/03/2009	750	<RLOD	< RLOD
FS09-0198	oysters	Kalang Adverse 1	K4-4	2/03/2009	500	<RLOD	< RLOD
FS09-0199	oysters	Kalang Adverse 1	K4-5	2/03/2009	1700	<RLOD	< RLOD
FS09-0200	oysters	Kalang Adverse 1	K5-1	2/03/2009	700	<RLOD	< RLOD
FS09-0201	oysters	Kalang Adverse 1	K5-2	2/03/2009	>18000	<RLOD	< RLOD
FS09-0202	oysters	Kalang Adverse 1	K5-3	2/03/2009	750	<RLOD	< RLOD
FS09-0203	oysters	Kalang Adverse 1	K5-4	2/03/2009	310	<RLOD	< RLOD
FS09-0204	oysters	Kalang Adverse 1	K5-5	2/03/2009	220	<RLOD	< RLOD
FS09-0205	oysters	Kalang Adverse 1	K7-1	2/03/2009	<20	<RLOD	< RLOD
FS09-0206	oysters	Kalang Adverse 1	K7-2	2/03/2009	<20	<RLOD	< RLOD
FS09-0207	oysters	Kalang Adverse 1	K7-3	2/03/2009	<20	<RLOD	< RLOD
FS09-0208	oysters	Kalang Adverse 1	K7-4	2/03/2009	40	<RLOD	< RLOD
FS09-0209	oysters	Kalang Adverse 1	K7-5	2/03/2009	70	<RLOD	< RLOD
FS09-0210	oysters	Kalang Adverse 1	K3-1	2/03/2009	2200	<RLOD	< RLOD
FS09-0211	oysters	Kalang Adverse 1	K3-2	2/03/2009	750	<RLOD	< RLOD
FS09-0212	oysters	Kalang Adverse 1	K3-3	2/03/2009	500	<RLOD	< RLOD
FS09-0213	oysters	Kalang Adverse 1	K3-4	2/03/2009	500	<RLOD	< RLOD
FS09-0214	oysters	Kalang Adverse 1	K3-5	2/03/2009	1600	<RLOD	< RLOD
FS09-0215	oysters	Kalang Adverse 1	K6-1	2/03/2009	110	<RLOD	< RLOD
FS09-0216	oysters	Kalang Adverse 1	K6-2	2/03/2009	5400	<RLOD	< RLOD
FS09-0217	oysters	Kalang Adverse 1	K6-3	2/03/2009	200	<RLOD	< RLOD
FS09-0218	oysters	Kalang Adverse 1	K6-4	2/03/2009	310	<RLOD	< RLOD
FS09-0219	oysters	Kalang Adverse 1	K6-5	2/03/2009	750	<RLOD	< RLOD
FS09-0220	oysters	Kalang Adverse 1	K1-1	2/03/2009	700	<RLOD	< RLOD
FS09-0221	oysters	Kalang Adverse 1	K1-2	2/03/2009	220	<RLOD	< RLOD
FS09-0222	oysters	Kalang Adverse 1	K1-3	2/03/2009	310	<RLOD	< RLOD
FS09-0223	oysters	Kalang Adverse 1	K1-4	2/03/2009	2200	<RLOD	< RLOD
FS09-0224	oysters	Kalang Adverse 1	K1-5	2/03/2009	3500	<RLOD	< RLOD
FS09-0225	oysters	Kalang Adverse 1	K8-1	2/03/2009	500	<RLOD	< RLOD
FS09-0226	oysters	Kalang Adverse 1	K8-2	2/03/2009	1100	<RLOD	< RLOD
FS09-0227	oysters	Kalang Adverse 1	K8-3	2/03/2009	1400	<RLOD	< RLOD
FS09-0228	oysters	Kalang Adverse 1	K8-4	2/03/2009	50	<RLOD	< RLOD
FS09-0229	oysters	Kalang Adverse 1	K8-5	2/03/2009	310	<RLOD	< RLOD
FS09-0230	oysters	Kalang Adverse 1	X1	2/03/2009	3500	<RLOD	< RLOD
FS09-0232	oysters	Kalang Adverse 1	X2	2/03/2009	500	<RLOD	< RLOD

Laboratory Number	Sample Type	Kalang Sample Type	Kalang Sample Site	Collected	<i>E coli</i> MPN/100g	NoVGI	NoV GII
FS09-1161	oysters	Kalang routine 2	K4-1	26/03/2009	70	<RLOD	< RLOD
FS09-1162	oysters	Kalang routine 2	K4-2	26/03/2009	70	<RLOD	< RLOD
FS09-1163	oysters	Kalang routine 2	K4-3	26/03/2009	20	<RLOD	< RLOD
FS09-1164	oysters	Kalang routine 2	K4-4	26/03/2009	70	<RLOD	< RLOD
FS09-1165	oysters	Kalang routine 2	K4-5	26/03/2009	<20	<RLOD	< RLOD
FS09-1166	oysters	Kalang routine 2	K5-1	26/03/2009	20	<RLOD	< RLOD
FS09-1167	oysters	Kalang routine 2	K5-2	26/03/2009	<20	<RLOD	< RLOD
FS09-1168	oysters	Kalang routine 2	K5-3	26/03/2009	<20	<RLOD	< RLOD
FS09-1169	oysters	Kalang routine 2	K5-4	26/03/2009	1300	<RLOD	< RLOD
FS09-1170	oysters	Kalang routine 2	K5-5	26/03/2009	40	<RLOD	< RLOD
FS09-1171	oysters	Kalang routine 2	K7-1	26/03/2009	20	<RLOD	< RLOD
FS09-1172	oysters	Kalang routine 2	K7-2	26/03/2009	220	<RLOD	< RLOD
FS09-1173	oysters	Kalang routine 2	K7-3	26/03/2009	2400	<RLOD	< RLOD
FS09-1174	oysters	Kalang routine 2	K7-4	26/03/2009	310	<RLOD	< RLOD
FS09-1175	oysters	Kalang routine 2	K7-5	26/03/2009	220	<RLOD	< RLOD
FS09-1176	oysters	Kalang routine 2	K3-1	26/03/2009	700	<RLOD	< RLOD
FS09-1177	oysters	Kalang routine 2	K3-2	26/03/2009	20	<RLOD	< RLOD
FS09-1178	oysters	Kalang routine 2	K3-3	26/03/2009	110	<RLOD	< RLOD
FS09-1179	oysters	Kalang routine 2	K3-4	26/03/2009	40	<RLOD	< RLOD
FS09-1180	oysters	Kalang routine 2	K3-5	26/03/2009	<20	<RLOD	< RLOD
FS09-1181	oysters	Kalang routine 2	K6-1	26/03/2009	<20	<RLOD	< RLOD
FS09-1182	oysters	Kalang routine 2	K6-2	26/03/2009	40	<RLOD	< RLOD
FS09-1183	oysters	Kalang routine 2	K6-3	26/03/2009	50	<RLOD	< RLOD
FS09-1184	oysters	Kalang routine 2	K6-4	26/03/2009	20	<RLOD	< RLOD
FS09-1185	oysters	Kalang routine 2	K6-5	26/03/2009	<20	<RLOD	< RLOD
FS09-1191	oysters	Kalang routine 2	K8-1	26/03/2009	110	<RLOD	< RLOD
FS09-1192	oysters	Kalang routine 2	K8-2	26/03/2009	<20	<RLOD	< RLOD
FS09-1193	oysters	Kalang routine 2	K8-3	26/03/2009	70	<RLOD	< RLOD
FS09-1194	oysters	Kalang routine 2	K8-4	26/03/2009	160	<RLOD	< RLOD
FS09-1195	oysters	Kalang routine 2	K8-5	26/03/2009	<20	<RLOD	< RLOD
FS09-1186	oysters	Kalang routine 2	K1-1	26/03/2009	110	<b>CT 42</b>	< RLOD
FS09-1187	oysters	Kalang routine 2	K1-2	26/03/2009	40	<RLOD	< RLOD
FS09-1188	oysters	Kalang routine 2	K1-3	26/03/2009	50	<RLOD	< RLOD
FS09-1189	oysters	Kalang routine 2	K1-4	26/03/2009	<20	<b>CT 38.5</b>	< RLOD
FS09-1190	oysters	Kalang routine 2	K1-5	26/03/2009	70	<RLOD	< RLOD
FS09-1471	oysters	Kalang Routine 3	K4-1	5/08/2009	<20	<b>41</b>	< RLOD
FS09-1472	oysters	Kalang Routine 3	K4-2	5/08/2009	<20	<b>41</b>	< RLOD

FS09-1473	oysters	Kalang Routine 3	K4-3	5/08/2009	20	<RLOD	< RLOD
FS09-1474	oysters	Kalang Routine 3	K4-4	5/08/2009	<20	<b>41</b>	< RLOD
FS09-1475	oysters	Kalang Routine 3	K4-5	5/08/2009	40	< RLOD	< RLOD
FS09-1476	oysters	Kalang Routine 3	K7-1	5/08/2009	40	<RLOD	< RLOD
FS09-1477	oysters	Kalang Routine 3	K7-2	5/08/2009	20	<RLOD	< RLOD
FS09-1478	oysters	Kalang Routine 3	K7-3	5/08/2009	20	<b>42</b>	< RLOD
FS09-1479	oysters	Kalang Routine 3	K7-4	5/08/2009	<20	<RLOD	< RLOD
FS09-1480	oysters	Kalang Routine 3	K7-5	5/08/2009	<20	<b>42</b>	< RLOD
FS09-1481	oysters	Kalang Routine 3	K3-1	5/08/2009	<20	<b>43</b>	< RLOD
FS09-1482	oysters	Kalang Routine 3	K3-2	5/08/2009	40	<RLOD	< RLOD
FS09-1483	oysters	Kalang Routine 3	K3-3	5/08/2009	<20	<RLOD	< RLOD
FS09-1484	oysters	Kalang Routine 3	K3-4	5/08/2009	20	<RLOD	< RLOD
FS09-1485	oysters	Kalang Routine 3	K3-5	5/08/2009	<20	<RLOD	< RLOD
						<RLOD	
FS09-1486	oysters	Kalang Routine 3	K6-1	5/08/2009	20	<RLOD	< RLOD
FS09-1487	oysters	Kalang Routine 3	K6-2	5/08/2009	20	<RLOD	< RLOD
FS09-1488	oysters	Kalang Routine 3	K6-3	5/08/2009	20	<RLOD	< RLOD
FS09-1489	oysters	Kalang Routine 3	K6-5	5/08/2009	20	<b>44</b>	< RLOD
FS09-1490	oysters	Kalang Routine 3	N5 - 5	5/08/2009	<20	<b>43</b>	< RLOD
FS09-1491	oysters	Kalang Routine 3	K8-1	5/08/2009	90	<RLOD	< RLOD
FS09-1492	oysters	Kalang Routine 3	K8-2	5/08/2009	<20	<b>41</b>	< RLOD
FS09-1493	oysters	Kalang Routine 3	K8-3	5/08/2009	20	<b>41</b>	< RLOD
FS09-1494	oysters	Kalang Routine 3	K8-4	5/08/2009	110	<b>44</b>	< RLOD
FS09-1495	oysters	Kalang Routine 3	K8-5	5/08/2009	20	<RLOD	< RLOD
FS09-1496	oysters	Kalang Routine 3	K1-1	5/08/2009	<20	<b>42</b>	< RLOD
FS09-1497	oysters	Kalang Routine 3	K1-2	5/08/2009	40	<RLOD	< RLOD
FS09-1498	oysters	Kalang Routine 3	K1-3	5/08/2009	40	<RLOD	< RLOD
FS09-1499	oysters	Kalang Routine 3	K1-4	5/08/2009	<20	<RLOD	< RLOD
FS09-1500	oysters	Kalang Routine 3	K1-5	5/08/2009	70	<RLOD	< RLOD
FS09-1501	oysters	Urunga STP	Influent bioaccumulated oysters	5/08/2009	NT	<RLOD	<b>35.9</b>
FS09-1511	oysters	Kalang Routine 4	K4-1	17/09/2009	40	<RLOD	< RLOD
FS09-1512	oysters	Kalang Routine 4	K4-2	17/09/2009	110	<RLOD	< RLOD
FS09-1513	oysters	Kalang Routine 4	K4-3	17/09/2009	70	<RLOD	< RLOD
FS09-1514	oysters	Kalang Routine 4	K4-4	17/09/2009	20	<RLOD	< RLOD
FS09-1515	oysters	Kalang Routine 4	K4-5	17/09/2009	200	<RLOD	< RLOD
FS09-1516	oysters	Kalang Routine 4	K5-1	17/09/2009	220	<RLOD	< RLOD
FS09-1517	oysters	Kalang Routine 4	K5-2	17/09/2009	40	<RLOD	< RLOD
FS09-1518	oysters	Kalang Routine 4	K5-3	17/09/2009	20	<RLOD	< RLOD
FS09-1519	oysters	Kalang Routine 4	K5-4	17/09/2009	20	<RLOD	< RLOD
FS09-1520	oysters	Kalang Routine 4	K7-1	17/09/2009	70	<RLOD	< RLOD

FS09-1521	oysters	Kalang Routine 4	K7-2	17/09/2009	<20	<RLOD	< RLOD
FS09-1522	oysters	Kalang Routine 4	K7-3	17/09/2009	20	<RLOD	< RLOD
FS09-1523	oysters	Kalang Routine 4	K7-4	17/09/2009	50	<RLOD	< RLOD
FS09-1524	oysters	Kalang Routine 4	K3-1	17/09/2009	20	<RLOD	< RLOD
FS09-1525	oysters	Kalang Routine 4	K3-2	17/09/2009	40	<RLOD	< RLOD
FS09-1526	oysters	Kalang Routine 4	K3-3	17/09/2009	<20	<RLOD	< RLOD
FS09-1527	oysters	Kalang Routine 4	K3-4	17/09/2009	<20	<RLOD	< RLOD
FS09-1528	oysters	Kalang Routine 4	K3-5	17/09/2009	40	<RLOD	< RLOD
FS09-1529	oysters	Kalang Routine 4	K6-1	17/09/2009	<20	<RLOD	< RLOD
FS09-1530	oysters	Kalang Routine 4	K6-2	17/09/2009	70	<RLOD	< RLOD
FS09-1531	oysters	Kalang Routine 4	K6-3	17/09/2009	20	<RLOD	< RLOD
FS09-1532	oysters	Kalang Routine 4	K6-4	17/09/2009	20	<RLOD	< RLOD
FS09-1533	oysters	Kalang Routine 4	K1-1	17/09/2009	20	<RLOD	< RLOD
FS09-1534	oysters	Kalang Routine 4	K1-2	17/09/2009	20	<RLOD	< RLOD
FS09-1535	oysters	Kalang Routine 4	K1-3	17/09/2009	110	<RLOD	< RLOD
FS09-1536	oysters	Kalang Routine 4	K1-4	17/09/2009	160	<RLOD	< RLOD
FS09-1537	oysters	Kalang Routine 4	K1-5	17/09/2009	200	<RLOD	< RLOD
FS09-1538	oysters	Kalang Routine 4	K8-1	17/09/2009	110	<RLOD	< RLOD
FS09-1539	oysters	Kalang Routine 4	K8-2	17/09/2009	200	<RLOD	< RLOD
FS09-1540	oysters	Kalang Routine 4	K8-3	17/09/2009	110	<RLOD	< RLOD
FS09-1541	oysters	Kalang Routine 4	K8-4	17/09/2009	20	<RLOD	< RLOD
FS09-1542	oysters	Kalang Routine 4	K8-5	17/09/2009	40	<RLOD	< RLOD
FS09-1543	oysters	Kalang Routine 4	Lagoon	17/09/2009	20	<RLOD	< RLOD

# AppendixA:5

Run number	High 1000	Medium 100	Low Ct	Low/2 Ct	Low/4 Ct	Low/10 Ct
	28.49	32.05	35			
		33.61	33.88			
		31.94	36.48			
		33.37	35.96			
		33.04	33.68			
		33.08	37.44			
		32.01	35.68			
		32.74	35			
test 1a	29.1	32.42	35.96	37.63	39.09	
			36.04	36.38	38.26	39.02
test 1b			36.33	36.6	38.49	39.56
			36.77	37.04	37.82	38.65
test 1c			36.37	37.17	37.59	39.4
			36.37	38	38.45	39.16
test 1d			35.69	36.53	39.02	
AVERAGE t1						39.1
test 2a	28.91	33.03	37.64	36.39	40.87	40.4
			36.58	37.31	40.37	40.24
test 2b			36.81	37.16	38.86	38.23
			36.68	36.8	37.49	39.57
test 2c			35.75	37.08	37.77	38.88
			35.86	36.44	38.25	38.33
test 2d			36.83	37.32		37.07
			37.2	36.28	37.96	38.48
AVERAGE t2						38.9
test 3a	28.78	32.33	35.33	37.46	37.3	38.17
			34.92	36.09	37.42	37.58
test 3b			35.59	35.8	37.44	38.04
			35.51	36.81	37.65	37.09
test 3c			35.39	36.62	40.01	38.22
			33.68	36.26	37.21	37.93
test 3d			36.1	36.98	37.05	39.41
			37.55	37.53	38.55	39.34
Average t3						38.2
t1, t2,t3 averaged to C <sub>T</sub>						35.8

**Figure A.5:** Calculations of NoV GI RLOD. C<sub>T</sub> of NoV GI RNA standard Low diluted to 1/10



# Appendix A:6 Chapter 5. Results for individual PCR results in genome copies/ g shellfish gut

Sampling days	5	12	20	28	34	42	48
Distance from pump station							
<b>50m</b>	190040	120360	63840	14680	1280	10360	<260
	199840	140960	61360	12000	<260	<260	<260
	143760	103920	64760	23880	<260	<260	<260
	2800880	77760	52040	42400	<260	<260	<260
<b>650m</b>	93360	30680	1000	<260	<260	<260	
	151560	20720	<260	3980	<260	<260	
	75280	22520	<260	420	<260	<260	
	127200	84440	<260	<260	<260	<260	
<b>1930m</b>	87760	<260	<260	<260	<260		
	246200	<260	<260	<260	<260		
	50760	<260	<260	<260	<260		
	12480	<260	<260	<260	<260		
<b>5290m</b>	160	<260	<260	<260	<260		
	520	<260	<260	<260	<260		
	<260	<260	<260	<260	<260		
	<260	<260	<260	<260	<260		
<b>6820m</b>	<260	<260	<260	<260	<260		
	<260	<260	<260	<260	<260		
	<260	<260	<260	<260	<260		
	<260	<260	<260	<260	<260		
<b>8530m</b>	24	50040	120	<260	<260		
	1840	32040	4840	280	<260		

## Appendix A:7 Best practice regulatory approaches to onsite sewage system management (293)

### Statements of Authorship and Published papers

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## D Best Practice Regulatory Approaches to Onsite Sewage System Management

Onsite sewage management systems (onsite systems) are regulated by councils through approvals issued under section 68 of the *Local Government Act 1993* (NSW) (the LG Act). In Chapter 5 of our Report we considered options to streamline section 68 approvals for low risk activities to reduce costs to business and the community. In this Appendix, we identify some examples of 'best practice' regulation by councils in relation to onsite systems. Installation and operation of onsite systems are high risk activities, as systems which are not properly installed, maintained and operated can pose significant public health and environmental risks.

Onsite systems are sewage treatment and disposal facilities installed at premises which are not connected to a reticulated sewerage system (ie, generally unsewered areas). These are typically household septic tanks and aerated wastewater treatment systems installed by the landowner.<sup>755</sup>

The largest number of section 68 approvals granted or renewed by councils each year is for onsite systems.<sup>756</sup> According to our recent licence survey, there were a total of 93,275 approvals to operate an onsite system in force during 2011-2012.<sup>757</sup> However, the number of systems in NSW has previously been estimated to be over 284,000.<sup>758</sup>

Given the large number of onsite system approvals and the need for ongoing regulation to protect public health and the environment, the broader adoption of best practice regulatory approaches in this area has the potential to reduce costs and provide benefits to councils, businesses and the community.

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<sup>755</sup> In 2001, standard septic tanks represented about 80% of onsite systems in NSW: Division of Local Government, *On-site Sewage Management Risk Assessment System Handbook*, (Draft) April 2001 (OSRAS Handbook), p 2-9, available at [http://www.dlg.nsw.gov.au/dlg/dlghome/dlg\\_osras.asp](http://www.dlg.nsw.gov.au/dlg/dlghome/dlg_osras.asp), accessed on 20 November 2013.

<sup>756</sup> According to our licence survey for the period 1 July 2011-30 June 2012, councils granted 25,580 and renewed 24,645 approvals to operate an onsite system: IPART, *Licensing Survey, Regulation Review – Licence Rationale and Design*, unpublished.

<sup>757</sup> Ibid.

<sup>758</sup> Kenway, S and Irvine, R, *Sewage Pollution Risk Assessment for Environmental Health*, Conference Proceedings Environmental Health Conference 2001, Bathurst, 11-12 September 2001, p 1, available at <http://www.dlg.nsw.gov.au/dlg/dlghome/documents/Information/Environmental%20Health%20Bathurst%202001.pdf>, accessed on 2 December 2013.

## D.1 Background

### D.1.1 Why regulate onsite systems?

In 2000, the Division of Local Government (DLG) estimated that around 70% of systems failed to meet environmental and health protection standards.<sup>759</sup> Failing onsite systems can release sewage into the environment, seeping into and contaminating waterways, which may spread disease or lead to environmental degradation. This is of particular concern when systems are within drinking water catchments or near areas with commercial aquaculture interests (such as oyster farming).<sup>760</sup> The cumulative effects of numerous failing systems can be significant.<sup>761</sup> For example, in 1997, over 4 hundred people were ill and one person died after eating oysters from Wallis Lake that were contaminated with the Hepatitis A virus. The exact source of the virus was never identified, but available evidence indicated the presence of faulty onsite systems which leaked raw sewage into the waterway which fed into Wallis Lake.<sup>762</sup> Following the Wallis Lakes incident, the requirement to obtain an approval to operate was imposed.

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<sup>759</sup> Division of Local Government, *Easy Septic Guide*, 2000, p 7, available at <http://www.dlg.nsw.gov.au/dlg/dlghome/documents/information/ssguide.pdf>, accessed on 2 December 2013.

<sup>760</sup> NSW Parliament, Legislative Assembly, Committee on Environment and Regulation, *Inquiry into the Regulation of Domestic Wastewater*, Report 1/55, November 2012 (Domestic Wastewater Inquiry Report), pp 17-19, available at [http://www.parliament.nsw.gov.au/prod/parlment/committee.nsf/0/54fe1a9d8f5b5843ca257b73002682e9/\\$FILE/Report%20on%20the%20Regulation%20of%20Domestic%20Wastewater%20FINAL.pdf](http://www.parliament.nsw.gov.au/prod/parlment/committee.nsf/0/54fe1a9d8f5b5843ca257b73002682e9/$FILE/Report%20on%20the%20Regulation%20of%20Domestic%20Wastewater%20FINAL.pdf), accessed on 25 November 2013.

<sup>761</sup> OSRAS Handbook, p 2-1.

<sup>762</sup> Domestic Wastewater Inquiry Report, p 17.

### D.1.2 Who regulates onsite systems?

**Councils** have the primary regulatory role for licensing onsite systems. Councils are required to manage the cumulative impacts of pollution from sewage in their local government area, which includes responsibility for approving onsite systems and monitoring their ongoing performance.<sup>763</sup> Councils are required to keep an up-to-date register of all onsite systems in their area.<sup>764</sup> Councils are also encouraged to develop and implement sewage management policies.<sup>765</sup> The LG Act allows councils to charge a fee for approval applications or renewals, and for undertaking inspections to fulfil their ongoing monitoring role.<sup>766</sup>

**NSW Health** is responsible for accrediting the design of onsite systems generally available for purchase by households (ie, premises normally occupied by no more than 10 persons).<sup>767</sup>

NSW Health Certificates of Accreditation require periodic servicing for certain systems which pose higher risks than other systems due to using more complicated technology.<sup>768</sup> For example, quarterly servicing by a service contractor is required for Aerated Wastewater Treatment Systems (AWTS).<sup>769</sup> The servicing can be undertaken either by a representative of the system manufacturer / distributor, or a service contractor “acceptable” to the council.<sup>770</sup> Councils impose this servicing requirement on the landowner as a condition of the section 68 approval.<sup>771</sup>

<sup>763</sup> OSRAS Handbook, pp 2-12.

<sup>764</sup> *Local Government Act 1993* (NSW), section 113.

<sup>765</sup> Division of Local Government, *The Environmental and Health Protection Guidelines: On-Site Sewage management for Single Households*, January 1998 (Silver Book), p 10 and Section 2, available at <http://www.dlg.nsw.gov.au/DLG/Documents/information/onsite.pdf>, accessed on 19 November 2013. City of Sydney Council, submission to Domestic Wastewater Inquiry, 2012, p 1, available at <https://www.parliament.nsw.gov.au/prod/parlment/committee.nsf/0/C14732C8E8F5535DCA2579980078CC7B>, accessed on 19 November 2013.

<sup>766</sup> *Local Government Act 1993* (NSW), sections 80, 107 and 608.

<sup>767</sup> *Local Government (General) Regulation 2005* (NSW), clauses 40-41.

<sup>768</sup> NSW Health AWTS Certificate, as discussed in Division of Local Government, *Sewage and Wastewater*, available at [http://www.dlg.nsw.gov.au/dlg/dlghome/dlg\\_policyindex.asp?areaindex=PLCYADV&documenttype=8&mi=6&ml=6&paid=33](http://www.dlg.nsw.gov.au/dlg/dlghome/dlg_policyindex.asp?areaindex=PLCYADV&documenttype=8&mi=6&ml=6&paid=33), accessed on 18 November 2013.

<sup>769</sup> See for example NSW Health Certificates of accreditation for various AWTS systems, available at [http://www.health.nsw.gov.au/environment/domesticwastewater/accreditations/awts\\_013.pdf](http://www.health.nsw.gov.au/environment/domesticwastewater/accreditations/awts_013.pdf) and [http://www.health.nsw.gov.au/environment/domesticwastewater/accreditations/awts\\_007.pdf](http://www.health.nsw.gov.au/environment/domesticwastewater/accreditations/awts_007.pdf), accessed on 18 November 2013.

<sup>770</sup> NSW Health AWTS Certificate, as discussed in Division of Local Government, *Sewage and Wastewater*, available at [http://www.dlg.nsw.gov.au/dlg/dlghome/dlg\\_policyindex.asp?areaindex=PLCYADV&documenttype=8&mi=6&ml=6&paid=33](http://www.dlg.nsw.gov.au/dlg/dlghome/dlg_policyindex.asp?areaindex=PLCYADV&documenttype=8&mi=6&ml=6&paid=33), accessed on 18 November 2013.

<sup>771</sup> For example, Port Macquarie-Hastings Council passes on the condition in the s68 approval to operate: Personal communication, Port Macquarie-Hastings Council, Email to IPART, 6 September 2013.

The Table below outlines the regulatory framework for the majority of onsite systems, being those used by households.

**Table D.1 Regulatory process for onsite systems**

Regulatory step	Responsible body	Low risk technology	High risk technology (eg, AWTs)
Accreditation (of system design and manufacture) <sup>a</sup>	NSW Health	✓	✓
s68 Approval to Install issued to landowner	Council	✓	✓
One-off Inspection (ensuring system installed in accordance with approval) <sup>b</sup>	Council	✓	✓
s68 Approval to Operate issued to landowner (ongoing approval renewed at intervals determined by council)	Council	✓	✓
Periodic servicing of system <sup>c</sup>	Service contractor	X	✓
Periodic inspections of system (to ensure system continuing to operate properly) <sup>d</sup>	Council	✓	✓

<sup>a</sup> *Local Government (General) Regulation 2005* (NSW), clauses 40-41.

<sup>b</sup> *Local Government (General) Regulation 2005* (NSW), clause 34. Port Macquarie-Hastings Council, Email to IPART, 6 September 2013.

<sup>c</sup> Only high risk technologies (represented by the final column must be serviced periodically during their operation. For example, NSW Health requires quarterly servicing by a service contractor for Aerated Wastewater Treatment Systems (AWTSs):  
[http://www.health.nsw.gov.au/environment/domesticwastewater/accreditations/awts\\_013.pdf](http://www.health.nsw.gov.au/environment/domesticwastewater/accreditations/awts_013.pdf) and  
[http://www.health.nsw.gov.au/environment/domesticwastewater/accreditations/awts\\_007.pdf](http://www.health.nsw.gov.au/environment/domesticwastewater/accreditations/awts_007.pdf), accessed on 18 November 2013.

<sup>d</sup> Division of Local Government, *On-site sewage management*, available at  
<http://www.dlg.nsw.gov.au/dlg/dlghome/PublicTopicsIndex.asp?mi=0&ml=10&id=10>, accessed on 26 November 2013.

DLG also has an advisory role in this area. It develops guidance material for councils and for onsite system operators. The key guidance document developed, in collaboration with other key State agencies with responsibilities in this area, is the 1998 *Environmental and Health Protection Guidelines: On-Site Sewage Management for Single Households* (the 'Silver Book' or 'Silver Bullet').<sup>772</sup> These are the technical standards used in the regulation of onsite systems. DLG also provides other separate guidance material, such as:

- ▼ a draft handbook on an onsite sewage risk assessment system (OSRAS Handbook), using spatial analysis technology (Geographic Information Systems (GIS)) to assess and map the likelihood or hazard of onsite system failure in varying circumstances<sup>773</sup>
- ▼ a handbook to assist councils to develop an information management system for onsite systems<sup>774</sup>
- ▼ model conditions for approval to operate an onsite system, for use in section 68 approvals<sup>775</sup>
- ▼ easy septic guide for householders<sup>776</sup>
- ▼ general website information for councils and system operators.<sup>777</sup>

<sup>772</sup> The Silver Book is available at <http://www.dlg.nsw.gov.au/DLG/Documents/information/onsite.pdf>. In 2004, DLG indicated that the Silver Book and OSRAS Handbook were to be revised that year: Division of Local Government, *Circular to Councils, 04/37*, 30 July 2004, available from <http://www.dlg.nsw.gov.au/dlg/dlghome/documents/Circulars/04-37.pdf>, accessed on 26 November 2013. Revised documents have not been released.

<sup>773</sup> OSRAS Handbook, pp 1-2, 1-3.

<sup>774</sup> Department of Local Government, *On-Site Sewage Information Management System*, available from <http://www.dlg.nsw.gov.au/DLG/Documents/information/SSIMS.pdf>, accessed on 25 November 2013.

<sup>775</sup> Division of Local Government, *Model Conditions to Operate an Onsite Sewage System*, available at [http://www.dlg.nsw.gov.au/dlg/dlghome/dlg\\_policyindex.asp?areaindex=PLCYADV&documenttype=8&mi=6&ml=6&paid=33](http://www.dlg.nsw.gov.au/dlg/dlghome/dlg_policyindex.asp?areaindex=PLCYADV&documenttype=8&mi=6&ml=6&paid=33), accessed on 20 November 2013.

<sup>776</sup> Department of Local Government, *Easy Septic Guide*, 2000, available from <http://www.dlg.nsw.gov.au/dlg/dlghome/documents/information/ssguide.pdf>, accessed on 25 November 2013.

<sup>777</sup> Division of Local Government, *On-site sewage management*, April 2010, available at <http://www.dlg.nsw.gov.au/dlg/dlghome/PublicTopicsIndex.asp?mi=0&ml=10&id=10>, and *Sewage and Wastewater*, available at [http://www.dlg.nsw.gov.au/dlg/dlghome/dlg\\_policyindex.asp?areaindex=PLCYADV&documenttype=8&mi=6&ml=6&paid=33](http://www.dlg.nsw.gov.au/dlg/dlghome/dlg_policyindex.asp?areaindex=PLCYADV&documenttype=8&mi=6&ml=6&paid=33), accessed on 18 November 2013.

### D.1.3 Other relevant reviews

There are a number of reviews underway (or recently concluded) that have considered matters related to the regulation of onsite systems.

#### Urban Water Regulation Review

The Metropolitan Water Directorate is leading a joint review of the *Water Industry Competition Act 2006* (NSW) (WIC Act) and regulatory arrangements for water recycling under the LG Act.<sup>778</sup>

Onsite systems on single or dual occupancy dwellings, normally occupied by no more than 10 persons (ie, small-scale household systems), are exempt from regulation under the WIC Act.<sup>779</sup> Therefore, the Metropolitan Water Directorate's review has not considered these onsite systems.<sup>780</sup>

Our discussion of onsite systems in this Appendix is confined to the regulation by councils of *only* small-scale household systems.

#### Domestic Wastewater Inquiry

In 2011, the Committee on Environment and Regulation (a standing committee of the NSW Legislative Assembly) began an inquiry on the regulation of domestic wastewater issues in NSW, releasing a final report in November 2012.<sup>781</sup> The NSW Government released an official response in 2013, deferring a decision on certain recommendations until the completion of the Urban Water Regulation Review, Independent Local Government Review Panel and Local Government Acts Taskforce reviews.<sup>782</sup>

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<sup>778</sup> Department of Finance and Services, *Urban Water Regulation Review: Discussion Paper*, November 2012 (Urban Water Review Discussion Paper), p 22, available at <http://engage.haveyoursay.nsw.gov.au/document/show/541>, accessed on 8 November 2013.

<sup>779</sup> *Water Industry Competition (General) Regulation 2008* (NSW), Schedule 3, clause 9.

<sup>780</sup> Urban Water Review Discussion Paper, p 14.

<sup>781</sup> Domestic Wastewater Inquiry Report, available at: [http://www.parliament.nsw.gov.au/prod/parlament/committee.nsf/0/54fe1a9d8f5b5843ca257b73002682e9/\\$FILE/Report%20on%20the%20Regulation%20of%20Domestic%20Wastewater%20FINAL.pdf](http://www.parliament.nsw.gov.au/prod/parlament/committee.nsf/0/54fe1a9d8f5b5843ca257b73002682e9/$FILE/Report%20on%20the%20Regulation%20of%20Domestic%20Wastewater%20FINAL.pdf), accessed on 25 November 2013.

<sup>782</sup> NSW Government, *NSW Government Response to Report No. 1/55 of the Legislative Assembly Committee on Environment and Regulation - Inquiry into the Regulation of Domestic Wastewater*, 2013, available at [http://www.parliament.nsw.gov.au/prod/parlament/committee.nsf/0/54fe1a9d8f5b5843ca257b73002682e9/\\$FILE/Gov.%20resp.%20to%20Rep.%20on%20Regulation%20of%20Domestic%20%20Wastewater%20.pdf](http://www.parliament.nsw.gov.au/prod/parlament/committee.nsf/0/54fe1a9d8f5b5843ca257b73002682e9/$FILE/Gov.%20resp.%20to%20Rep.%20on%20Regulation%20of%20Domestic%20%20Wastewater%20.pdf), accessed on 6 November 2013.



## D.2 Best practice regulatory approaches

Stakeholders made a number of comments on this area of regulation, including:

- ▼ resourcing and capacity issues preventing councils from adequately regulating onsite systems<sup>783</sup>
- ▼ the value of implementing a risk-based approach to the regulation of onsite systems.<sup>784</sup>

Through our further research of this area, we identified best practice regulatory approaches that also address issues with:

- ▼ ineffective servicing by service contractors
- ▼ dual approvals for installing and operating onsite systems.

These issues and best practice approaches are discussed further below.

### D.2.1 Resourcing and capacity constraints

Councils have the regulatory powers to set performance standards, related maintenance and reporting requirements through approvals to operate, and to recover approval, renewal and inspection fees towards the cost of risk assessment and performance supervision.<sup>785</sup> However, stakeholders have indicated that due to resource constraints, some councils are unable to implement satisfactory inspection and compliance programs for onsite systems.<sup>786</sup>

Maintaining an ongoing inspection program can be very costly for councils.<sup>787</sup> As noted by Port Macquarie-Hastings Council:

Inspection procedures are ...at the discretion of local councils ... The extent of monitoring is usually directly related to the resources of the particular council.<sup>788</sup>

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<sup>783</sup> Liverpool Plains Shire Council's submission to IPART, October 2012.

<sup>784</sup> Wollondilly Shire Council's submissions to IPART's Licence Rationale and Design Review, December 2012; Port Macquarie-Hastings Council, Licence Rationale and Design Review, Public Roundtable, Transcript, pp 68-69.

<sup>785</sup> Division of Local Government, *Circular to Councils, 04/37*, 30 July 2004, available from <http://www.dlg.nsw.gov.au/dlg/dlghome/documents/Circulars/04-37.pdf>, accessed on 26 November 2013.

<sup>786</sup> For example, Liverpool Plains Shire Council's submission to IPART, October 2012, p 6.

<sup>787</sup> Personal communication, Telephone conversation between Whitehead & Associates Environmental Consultants and IPART, 9 August 2013.

<sup>788</sup> Port Macquarie-Hastings Council submission to Domestic Wastewater Inquiry, December 2011, p 2, available at <https://www.parliament.nsw.gov.au/prod/parlment/committee.nsf/0/7E3A7423864A67A2CA2579980078D419>, accessed on 6 November 2013.

Revenue policy for onsite system approvals and inspection fees is a matter for each council to determine (eg, the exact fee and what the money is used for).<sup>789</sup> Some councils find their current resources are insufficient to conduct the number of on-the-ground inspections needed.<sup>790</sup> Some councils disburse funds raised back into general revenue for the overall council, rather than dedicating fees for onsite system regulation and inspections.<sup>791</sup>

As the number of onsite systems in NSW is considerable, regulation of these systems can be a large impost on council's human resources. Moreover, Environmental Health Officers (EHOs) are generally responsible for a wide range of health matters (not just onsite systems).<sup>792</sup>

Pressure on staff resources could be exacerbated by the clustering of systems in certain geographical areas, which results in uneven resource implications across the State. This may affect council's capacity to adequately regulate and inspect systems. The Figure below demonstrates clustering of systems across council types.

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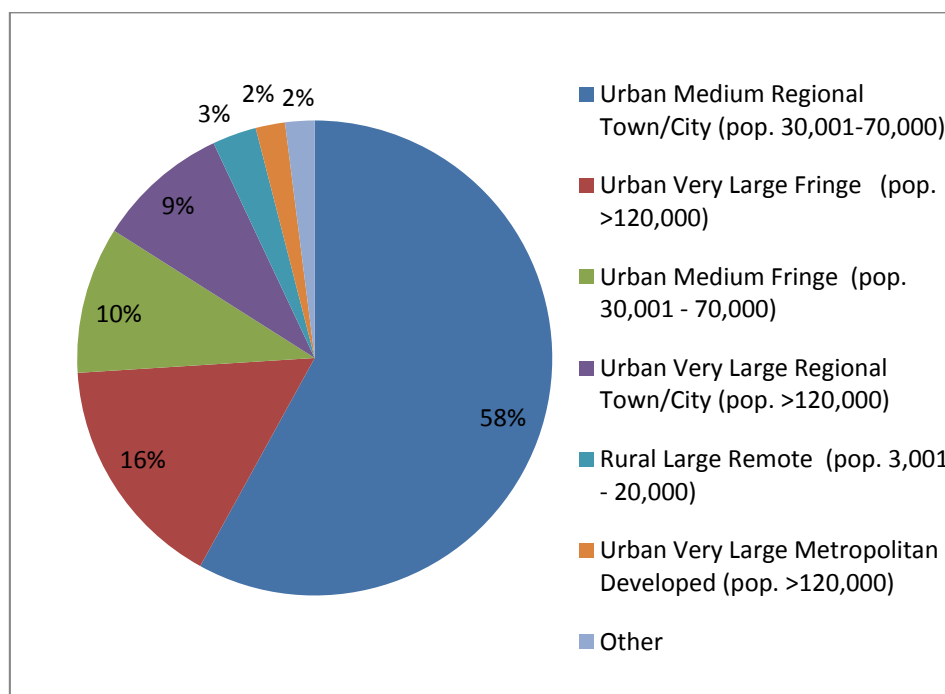
<sup>789</sup> Division of Local Government, *Sewage Management (SepticSafe Program) Update*, July 2004, at <http://www.dlg.nsw.gov.au/dlg/dlghome/documents/Circulars/04-37.pdf>, accessed 19 November 2013.

<sup>790</sup> Shoalhaven City Council submission to Domestic Wastewater Inquiry, December 2011, p 2, available at <https://www.parliament.nsw.gov.au/prod/parlment/committee.nsf/0/B43BAC42C67401FECA25799800795592>, accessed on 19 November 2013.

<sup>791</sup> Personal communication, Telephone conversation between Wollondilly Shire Council and IPART, 3 September 2013.

<sup>792</sup> Personal communication, Telephone conversation between Whitehead & Associates Environmental Consultants and IPART, 9 August 2013.

**Figure D.1 Distribution of Section 68 (c6) Approvals to Operate a System of Sewage Management, total in force 30 June 2012**



**Data source:** Analysis of IPART Review of Licence Rationale and Design local council licence survey data (2012). Definition of council types is from the IPART local government survey database, which groups councils based on geography and population size.

**Regional councils** issue the majority of approvals to operate (67%). Regional councils near waterways and with related industries (eg, tourism, aquaculture, oyster farming) were found to have implemented 'best practice' regulatory programs, due to the expertise gained with having large numbers of high risk onsite systems.<sup>793</sup>

**Urban fringe councils** issue 26% of approvals to operate. These councils often experience resource pressures due to rapid growth, impacting on their regulatory capacity.<sup>794</sup>

**Rural and remote councils** – while only 3% of approvals to operate in force were issued by rural-remote councils, these councils can lack the resources and expertise to undertake adequate regulation.<sup>795</sup> Such councils are responsible for large land masses, and can have high travel costs and limited budgets and staff.<sup>796</sup>

<sup>793</sup> For example, Eurobodalla Shire Council, Port Macquarie Hastings Council and Wagga Wagga City Council – see section D.2.2 for further details.

<sup>794</sup> Personal communications, Telephone conversation between Metropolitan Water Directorate and IPART, 29 July 2013; Telephone conversation between IPART Water and Regulation Review teams, 31 July 2013.

<sup>795</sup> Liverpool Plains Shire Council's submission to IPART, October 2012.

<sup>796</sup> Wentworth Shire Council submission to IPART, October 2013.

**Major metropolitan councils** issue a small percentage of approvals (2%). As a result these councils can lack the technical and regulatory experience to manage these systems properly.<sup>797</sup>

### D.2.2 Best Practice approach: Risk-based regulation

A number of councils have implemented risk-based regulation and revenue policies which enable better management of limited resources and more efficient regulation.

In one council, use of such a regulatory program reduced non-compliances (structural defects and/or unhealthy conditions) dramatically. In 2003, 75% of onsite systems within the Eurobodalla Shire Council's boundaries needed work. In 2011, this had reduced to only 15% of systems needing work.<sup>798</sup>

The Box below outlines the 2 key elements for best practice regulation in this area.

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<sup>797</sup> Personal communication, Telephone conversation between IPART Water and Regulation Review teams, 30 October 2013. For example, Randwick City Council has indicated it currently has only 5 systems approved to operate in its local government area: Personal communication, Randwick City Council, Email to IPART, 17 October 2013.

<sup>798</sup> Eurobodalla Shire Council submission to Domestic Wastewater Inquiry, December 2011, p 5, available at <https://www.parliament.nsw.gov.au/prod/parlment/committee.nsf/0/29D53433283AC38ACA25799800791291>, accessed on 19 November 2013.

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### Box D.1 Best practice regulation – 2 key elements

**Efficient sewage management revenue policies** - setting fees efficiently to recover costs, using the provisions of the LG Act to automate payment, and dedicating revenue to onsite system regulation.

**Risk-based, targeted approvals and inspections** - the use of appropriate risk frameworks to guide decision-making in setting approval/renewal durations and inspection frequency. This would (at minimum) include the following risk factors in any basic risk framework:<sup>a</sup>

- ▼ compliance history of applicant,
- ▼ volume of effluent system is capable of treating,
- ▼ location of system, including proximity to water, soil type and topography,
- ▼ concentration of systems,
- ▼ disposal area (land size, efficiency at processing), and
- ▼ risk/complexity of the technology of the system (ie, technology type).

<sup>a</sup> Personal communications, Meeting between IPART Water and Regulation Review teams, 31 July 2013; Telephone conversation between NSW Health and IPART, 9 August 2013; Email from NSW Health to IPART, 4 March 2014. For a more detailed risk assessment matrix, see Wagga Wagga City Council's *On-site Sewage Management Plan 2009-14*, 2009, pp13-14 & Appendix 1, available at [http://www.wagga.nsw.gov.au/\\_\\_data/assets/pdf\\_file/0017/2933/On\\_Site\\_Sewage\\_Management\\_Plan.pdf](http://www.wagga.nsw.gov.au/__data/assets/pdf_file/0017/2933/On_Site_Sewage_Management_Plan.pdf), accessed on 20 October 2013.

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The Table below highlights 3 examples of best practice approaches incorporating these key elements.

**Table D.2 Best practice regimes of councils in onsite system regulation**

	Risk-based approach to licencing?		Efficient revenue policy?	
	Approvals	Ongoing Inspections	Approvals	Inspections
Port Macquarie-Hastings Council <sup>a</sup>	Yes – initial section 68 approval issued for 5 years. After 5 years, system is inspected and risk rated. Approval must be renewed based on risk rating, every 1, 3 or 5 years (ie, high, medium or low risk). Licensees with positive compliance history may be rewarded with less frequent renewal periods.	Yes – inspection frequency corresponds to renewal period (eg, every 1, 3 or 5 years). Licensee can be re-rated to be inspected more frequently if a poor compliance history is demonstrated.	Flat approval fee. Council is considering a graduated fee scheme to encourage use of lowest-risk technologies (as per Wagga Wagga below). Uses s107A of LG Act to automatically renew and levy renewal fee on licensee's Rates Notices. Dedicates revenue to onsite system management.	Charges for initial inspection(s) under Approval to Install. Inspections under Approval to Operate are covered from flat approval fee.
Eurobodalla Shire Council <sup>b</sup>	Yes –section 68 approvals issued for 1, 2 or 5 years based on a risk assessment. Licensees with positive compliance history are rewarded with less frequent renewal periods.	Yes – inspection frequency corresponds to renewal period (eg, every 1, 2 or 5 years). Licensees with positive compliance history are rewarded with fewer inspections.	Flat approval fee. Dedicates revenue back to onsite system management.	Charges for inspections on user pays basis under s608 LG Act ('reasonable fee for service').
Wagga Wagga City Council <sup>c</sup>	Yes –section 68 approvals issued for 3, 6 or 12 months, or 1, 1-3, 3-5, 5, 5-10 or 10 years based on a risk assessment.	Yes - inspection frequency corresponds to renewal period. Can be exempt from inspections under very specific low-risk circumstances.	Graduated sliding scale of approval fees depending on risk of system technology, encouraging licensees to choose lowest risk system appropriate for their needs.	Charges for inspections on user pays basis.

<sup>a</sup> Personal communications, Port Macquarie-Hastings Council, Emails to IPART, 6 August 2013, 18 November 2013, 9 December 2013 and 13 March 2014.

<sup>b</sup> Eurobodalla Shire Council, *On Site Sewage Management Code of Practice*, p 9, available at <http://www.esc.nsw.gov.au/inside-council/council/council-policies/codes/2013-OSMS-CoP-24-Sept-13.pdf>, accessed on 3 December 2013; Personal communication, Telephone conversation between Eurobodalla Shire Council and IPART, 12 September 2013.

<sup>c</sup> Wagga Wagga City Council, *On-site Sewage Management Plan 2009-14*, 2009, pp 13-15, available at [http://www.wagga.nsw.gov.au/\\_data/assets/pdf\\_file/0017/2933/On\\_Site\\_Sewage\\_Management\\_Plan.pdf](http://www.wagga.nsw.gov.au/_data/assets/pdf_file/0017/2933/On_Site_Sewage_Management_Plan.pdf), accessed on 20 October 2013; Personal communication, Telephone conversation between Metropolitan Water Directorate and IPART, 25 July 2013.

Implementation of a risk-based approach to onsite system regulation represents 'best practice' by reducing costs to landowners who are 'good' operators or operate low risk systems through reduced approval, renewal and inspection fees. Implementing a risk-based approach allows better targeting of limited resources and results in more effective regulation. For example, more inspections/more frequent servicing of high risk systems or fewer inspections/less frequent servicing of low risk systems.<sup>799</sup> An alternate may be having reduced inspection requirements for systems with good compliance history. This has net benefits to the community through better protection of public health and the environment.

Attaching renewal and inspection fees to the annual Rates Notice (issued quarterly), rather than to a single lump sum invoice, is an approach that enables landowners to spread the costs of council onsite system inspections over the year.<sup>800</sup> It also gives owners the opportunity to pay by instalments if necessary,<sup>801</sup> as well as automatically renewing the approval.<sup>802</sup> We consider this is 'best practice' because it reduces the red tape imposed on the landowner, as they do not have to fill out renewal paperwork (the approval is taken to be renewed on the same terms). It also reduces resource pressures on the council by automating the renewal process. Dedication of these fees to onsite system management also ensures such programs are efficiently funded.

### D.2.3 Ineffective servicing by onsite system contractors

Stakeholders have raised a number of issues with the servicing of onsite systems undertaken by some private contractors, including:

- ▼ variable quality services<sup>803</sup>, and
- ▼ a lack of standardised information provided by service contractors<sup>804</sup>.

<sup>799</sup> The number of services (eg, annual, quarterly, etc.) and council inspections required for different systems is generally known at the outset, so people can choose a system with that in mind.

<sup>800</sup> Eurobodalla Shire Council, *PS13/09 Draft Onsite Sewage Management Code of Practice: Report to Policy and Strategy Committee Meeting of Eurobodalla Shire Council*, July 2013, pp 15-16, available at [http://www.esc.nsw.gov.au/inside-council/council/meetings/2013/july/policy-and-strategy-committee-meeting/130702\\_agenda.pdf](http://www.esc.nsw.gov.au/inside-council/council/meetings/2013/july/policy-and-strategy-committee-meeting/130702_agenda.pdf), accessed on 2 December 2013.

<sup>801</sup> Ibid.

<sup>802</sup> Personal communication, Telephone conversation between Port Macquarie-Hastings Council and IPART, 8 January 2013.

<sup>803</sup> See submissions from various councils to the Domestic Wastewater Inquiry, including: Shoalhaven City Council's submission, December 2011, p 6, available at <https://www.parliament.nsw.gov.au/prod/parlment/committee.nsf/0/B43BAC42C67401FEC A25799800795592>; and Eurobodalla Shire Council's submission, December 2011, p 9, available at <https://www.parliament.nsw.gov.au/prod/parlment/committee.nsf/0/29D53433283AC38ACA25799800791291>; accessed on 2 December 2013.

<sup>804</sup> See Bega Valley Shire Council submission to the Domestic Wastewater Inquiry, December 2011, available at <https://www.parliament.nsw.gov.au/prod/parlment/committee.nsf/0/772D844F5902DF43CA2579AD0076A2E9>, accessed on 2 December 2013.



### Best practice approach to variable quality servicing

Councils have indicated there is variable quality in contractor services. Since 1998, the market was opened to allow private contractors to conduct these services (rather than having to be serviced by the system's manufacturer).<sup>805</sup> There is no licencing or accreditation scheme for service contractors. Stakeholders have complained that the quality of servicing undertaken by contractors varies greatly. This is a cause for concern for system operators as they remain liable for any failures to comply with the conditions of the approval.<sup>806</sup>

Where contractors find issues or system faults, there can be limited incentives for documenting them in service reports, as the contractor is engaged and paid by the system operator (not the council). If the service report contains defects, service contractors could lose a revenue stream if operators prefer to look for "a more obliging service provider".<sup>807</sup> This can exacerbate the public health risk from potential system failure.

Some service contractors also undertake 'tick and flick' servicing, where the actual septic tank is not checked or the service contractor does not even access the property on which the system is situated.<sup>808</sup>

Councils can determine the "acceptability" of service contractors in their area by setting minimum criteria. Any service contractors operating in their area can then apply to the council for inclusion on their list of acceptable service contractors provided they meet the criteria.<sup>809</sup>

The Box below outlines an innovative current practice that addresses this issue using the current regulatory framework.<sup>810</sup>

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<sup>805</sup> BioSeptic (an aerated system manufacturer) submission to Domestic Wastewater Inquiry, December 2011, p 5, available at <https://www.parliament.nsw.gov.au/prod/parlment/committee.nsf/0/E64F94E8C160DE95CA25799800793A7A>, accessed on 18 November 2013.

<sup>806</sup> Personal communications, Telephone conversation between Port Macquarie-Hastings Council and IPART, 8 September 2013; Telephone conversation between Whitehead & Associates Environmental Consultants and IPART, 9 August 2013.

<sup>807</sup> BioSeptic submission to Domestic Wastewater Inquiry, December 2011, p 6; Personal communication, Port Macquarie-Hastings Council, Email to IPART, 31 October 2013.

<sup>808</sup> Personal communications, Telephone conversation between Port Macquarie-Hastings Council and IPART, 8 September 2013; Telephone conversation between Whitehead & Associates Environmental Consultants and IPART, 9 August 2013.

<sup>809</sup> Division of Local Government, *Sewage and Wastewater*, at [http://www.dlg.nsw.gov.au/dlg/dlghome/dlg\\_policyindex.asp?areaindex=PLCYADV&documenttype=8&mi=6&ml=6&paid=33](http://www.dlg.nsw.gov.au/dlg/dlghome/dlg_policyindex.asp?areaindex=PLCYADV&documenttype=8&mi=6&ml=6&paid=33), accessed 18 November 2013.

<sup>810</sup> We note the Domestic Wastewater Inquiry discusses the regulation of service contractors and makes recommendations for change to the existing regulatory framework in this area: Domestic Wastewater Inquiry Report, pp 42-45.



## Box D.2 Using regional groupings to set common service standards

Some councils have grouped together regionally to swap knowledge of contractors to address issues with variable quality services (for example, the Septic Tank Action Group (STAG) in the Hunter). STAG have jointly determined the acceptable criteria, in order to have a consistent, high standard for service contractors on a regional basis. This enhances consistency across council boundaries and raises the quality of services undertaken. This initiative has been supported as best practice by DLG, the Domestic Wastewater Inquiry and by NSW Health.

This practice assists in the management of service contractors and encourages cross-fertilization of effective onsite system management practices amongst councils, without imposing the extensive regulatory requirements of a formal licensing regime. The Domestic Wastewater Inquiry noted that Regional Organisations of Councils (ROCs) provide another model for regional collaboration in this area.

**Sources:** Division of Local Government, *Sewage and Wastewater*, at [http://www.dlg.nsw.gov.au/dlg/dlghome/dlg\\_policyindex.asp?areaindex=PLCYADV&documenttype=8&mi=6&ml=6&paid=33](http://www.dlg.nsw.gov.au/dlg/dlghome/dlg_policyindex.asp?areaindex=PLCYADV&documenttype=8&mi=6&ml=6&paid=33), accessed 18 November 2013; NSW Parliament, Legislative Assembly, Committee on Environment and Regulation, *Inquiry Into the Regulation of Domestic Wastewater*, Report 1/55, November 2012 (Domestic Wastewater Inquiry Report), pp 44-45, 50-51; Personal communication, Telephone conversation between NSW Health and IPART, 9 August 2013.

## Best practice approach to lack of standardised information reporting

Service contractors are to provide a copy of the service report to the system operator and the council (as well as retaining a copy for themselves).<sup>811</sup> There is currently no standard service report for contractors to use. As a result, the information provided can be highly variable and inconsistent.<sup>812</sup> Stakeholders have indicated that the interpretation of forms and data provided can be a time-consuming and expensive process.<sup>813</sup> Where key information required to assess risk is missing, councils are also more limited in their ability to proactively manage public health challenges associated with onsite systems.<sup>814</sup> This leads to additional resource pressures on councils, as it is estimated that some councils could deal with more than 16,000 reports per year.<sup>815</sup>

<sup>811</sup> NSW Health, Certificate of Accreditation, Aerated Wastewater Treatment System as per Personal communication, Port Macquarie-Hastings Council, Email to IPART, 5 November 2013.

<sup>812</sup> Personal communication, Telephone conversation between Port Macquarie-Hastings Council and IPART, 8 September 2013; see also Septic Tank Action Group (STAG) submission to Domestic Wastewater Inquiry, January 2012, p 3.

<sup>813</sup> Septic Tank Action Group (STAG) submission to Domestic Wastewater Inquiry, January 2012, p 3.

<sup>814</sup> Septic Tank Action Group (STAG) submission to Domestic Wastewater Inquiry, January 2012, p 3; Personal communication, Telephone conversation between Port Macquarie-Hastings Council and IPART, 8 September 2013.

<sup>815</sup> Personal communication, Telephone conversation between NSW Health and IPART, 9 August 2013.

To address these issues, the Domestic Wastewater Inquiry recommended that Fair Trading or DLG develop a common reporting standard and template to be submitted through a State Government electronic portal and that the reports should be filed on a common database that is accessible by all councils.<sup>816</sup>

Some councils or groups of councils have progressed work on such a template. The Box below provides one such example of a draft template developed by the Southern NSW Onsite System Special Interest Group ('Southern NSW SIG')<sup>817</sup>. Members of the Septic Tank Action Group (STAG)<sup>818</sup> believe there will be considerable efficiencies gained by using a template to streamline processes, to the benefit of councils, service contractors and system operators. They also envisage that an electronic format of a finalised template could be developed to further ease the regulatory burden of onsite system service reports.<sup>819</sup>

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<sup>816</sup> Domestic Wastewater Inquiry Report, pp vii and 46.

<sup>817</sup> The Southern NSW SIG is made up of many southern council Environmental Health Officers or EHOs, including Eurobodalla Shire Council and Bega Valley Shire Council.

<sup>818</sup> STAG is made up of many Central Coast and Mid-North Coast NSW Council EHOs, including Port Macquarie Hastings Council and Great Lakes Council.

<sup>819</sup> Personal communication, Port Macquarie Hastings Council, Email to IPART, 13 December 2013; Personal communication, Great Lakes Council, Email to IPART, 13 December 2013.

**Figure D.2 Possible Template for Contractors Inspecting Aerated Systems**

GENERIC SERVICE AGENT GENERIC ADDRESS GENERIC PHONE NUMBER ABN			
<b>Aerated Wastewater Treatment System (AWTS) SERVICE REPORT</b>			
Name/Owner/Occupier: _____			
Site Address: _____ Town / Suburb: _____ PC: _____			
Council Area: _____ Approval No: _____ System: _____			
System age: _____ Yrs:      Service cycle : 1 / 2 / 3 / 4      Date of service: ____/____/20____			
Rating System: G- Good    F – Fair    P- Poor			
<b>1. Septic tank</b>	<b>Condition</b>	<b>4. Irrigation Area</b>	<b>Condition</b>
Septic tank condition		Inline Filter check	N/A
Square junctions		Pipes Buried Movable	
Sludge depth	mm	Sprinklers/Drippers	
Desludge required	Y N	Working (number)	
Scum depth	mm	Subsurface	
Outlet filter	N/A	Signage (number)	
<b>2. Treatment tank</b>		Ponding	
Air valves working		Overspray	
Check & adjust Air supply		Bore on Property	
Scum in clarifier		<b>5. General</b>	
Skimmer working	N/A	Tanks & surrounds	
Clarifier clarity	mm	Odour	
Sludge depth in Clarifier	mm	Plans in Meter Box	Y/N
Sludge return Working	N/A Y/N	<b>6. Comments/Recommendations</b>	
Chlorine Contact tank clarity	mm		
Chlorine test	N/A mg/L		
pH test			
Dissolved Oxygen test	mg/L		
Chlorine tablets remaining	N/A		
Chlorine tablets added	N/A		
Flow orifice check			
<b>3. Electronic components</b>			
Air blower working	N/A Y/N	amps	
Air Filter check			
Air Pump working	N/A Y/N	amps	
Irrigation pump working	Y/N	amps	
Sludge return pump working	N/A Y/N	amps	
UV Working	Y/N/ N/A		
Timer check			
Leads & connectors			
Circuit Breakers/Fuses			
Low Air Alarm working	N/A *A/V Y/N		
Irrigation pump alarm working	A/V H/L o Y/N		
Alarm Panel	Int/Ext		
Note: * A/V = Audible/Visual			
Start time: .....am/pm      Finish time: .....am/pm			
Service technician 1: _____		Signed _____	
Service technician 2: _____		Signed _____	

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**Source:** Southern NSW Onsite System Special Interest Group - Personal communication, Eurobodalla Shire Council, Email to IPART, 18 September 2013.

#### D.2.4 Best practice approach to dual approvals

Prior to the Wallis Lakes incident, landowners only required an approval to install an onsite system. Following Wallis Lakes, an approval to operate was also required. This is the current situation.

The approval to operate requires regular renewal and ongoing council inspections, to ensure that a system continues to function properly over its lifetime.<sup>820</sup> Whereas an approval to install is not renewed by councils once the system is installed and operating.

However, stakeholders have indicated that landowners do not like having to apply for 2 approvals, as they do not understand why 2 approvals are necessary.<sup>821</sup> Some councils, such as Port Macquarie-Hastings Council, have started issuing the approval to install and approval to operate together as a package of approvals in the initial licence grant, in order to reduce paperwork for the system owner.<sup>822</sup>

The Box below outlines Port Macquarie-Hastings Council's approach.

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#### Box D.3 Issuing Both Approvals at Once - Port Macquarie-Hastings Council

Port Macquarie-Hastings Council issues a 5-year approval to install and a 5-year approval to operate together as a package. After the expiry of these initial approvals, systems are risk-rated to determine how often the approval to operate must be renewed and the system must be inspected.

Under clause 34 of the *Local Government (General) Regulation 2005*, a standard condition of an approval to install is that the system cannot be operated until the council has given notice in writing that it is satisfied the system has been installed in accordance with the approval. That is, the system owner cannot operate the system under the initial approval to operate until the council provides such notice, without being in breach of their approval to install.

This reduces costs to system owners by reducing processing times, dual provision of information, and delays (through processing both approvals at the one time).

**Sources:** Personal communications, telephone conversations between Port Macquarie-Hastings Council and IPART, 10 December 2013; Port Macquarie-Hastings Council, Email to IPART, 6 September 2013; *Local Government (General) Regulation 2005* (NSW), clause 34.

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<sup>820</sup> *Local Government Amendment (Miscellaneous) Act 2002* (NSW), No 40, Schedule 1 [11]-[13].

<sup>821</sup> Personal communications, Telephone conversation between Port Macquarie-Hastings Council and IPART, 8 September 2013; Telephone conversation between Eurobodalla Shire Council and IPART, 12 September 2013.

<sup>822</sup> Personal communication, Telephone conversation between Port Macquarie-Hastings Council and IPART, 10 December 2013.

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## **Paper 1: A survey of Australian oysters for the presence of human noroviruses**

Published in Food Microbiology, **44** (2014) 264-270

Presented in Chapter 4 and in the Appendix.

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**Paper 2: Spatial and temporal distribution of norovirus and *E. coli* in Sydney rock oysters following a sewage overflow into an estuary.**

Presented in Chapter 5

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**Paper 3: Evaluation of the risk management of Australian oyster harvest areas for the protection of the oyster consumer from NoV illness**

Presented in Chapter 6.

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Brake F.A., Ross T., Holds G., Kiermeier A., &  
McLeod C. 2014, A survey of Australian  
oysters for the presence of human  
noroviruses, Food microbiology 44, 264 –  
270